(54) Title: NOVEL ENZYME

(57) Abstract: A recombinant protein having luciferase activity and at least 60 % similarity to a wild-type luciferase wherein in the sequence of the enzyme, the amino acid residue corresponding to residue 357 in Photinus pyralis luciferase is mutated as compared to the corresponding wild-type luciferase, such that the luciferase enzyme is able to emit light at a different wavelength as compared to the corresponding wild-type luciferase and/or has enhanced thermostability as compared to the corresponding wild-type luciferase. In general, the residue corresponding to 357 in Photinus pyralis luciferase is changed from an acidic amino acid to a non-acidic amino acid and preferably an uncharged polar amino acid such as tyrosine. Mutant luciferases in accordance with the invention can produce a large (50nm) wavelength shift in emitted light and have good thermostability. The resultant colour shift can be reversed by addition of coenzyme A. These properties make the mutant particularly useful in a variety of assays.

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Novel Enzyme

The present invention relates to a novel protein, in particular
mutant luciferase enzymes which show distinctive properties as
5   compared to corresponding wild type enzyme, to DNA encoding
these proteins, to the use of these enzyme in assays and to
test kits containing them.

Firefly luciferase catalyses the oxidation of luciferin in the
10   presence of ATP, Mg^{2+} and molecular oxygen with the resultant
production of light. This reaction has a quantum yield of
about 0.88. The light emitting property has led to its use in
a wide variety of luminometric assays where ATP levels are
being measured. Examples of such assays include those which
15   are based upon the described in EP-B-680515 and WO 96/02665 but
many others are used routinely in laboratories.

Luciferase is obtainable directly from the bodies of insects,
in particular beetles such as fireflies or glow-worms.
20   Particular species from which luciferases have been obtained
include the Japanese GENJI or KEIKE fireflies, *Luciola cruciata*
and *Luciola lateralis*, the East European firefly *Luciola
mingrelica*, the North American firefly *Photinus pyralis* and the
glow-worm *Lampyris noctiluca*.
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However, since many of the genes encoding these enzymes have
been cloned and sequenced, they may also be produced using
recombinant DNA technology. Recombinant DNA sequences encoding
the enzymes are used to transform microorganisms such as *E.*
30   *coli* which then express the desired enzyme product.

The colour of the light emitted by these enzymes when used in
assays in the laboratory are broadly similar. It would be
helpful if the wavelength could be altered, either to be more
35   easily read by the specific detector, or for use in systems
where multiple reporters are required, for example to monitor
different events within the same sample. One way of
distinguishing reporter molecules is to utilise luciferase

molecules which emit light at distinct wavelengths. This may
be achieved by using reporter molecules comprising luciferases
derived from different species of beetle or glow-worm. An
alternative strategy however is to produce mutant luciferases
5   using recombinant DNA technology, so as to produce a variation
in the wavelength of the signal. Examples of such mutants are
provided in WO 95/18853.

Furthermore, the heat stability of wild and recombinant type
10   luciferases is such that they lose activity quite rapidly when
exposed to temperatures in excess of about 30°C, particularly
over 35°C. This instability causes problems when the enzyme is
used or stored at high ambient temperature, or if the assay is
effected under high temperature reaction conditions, for
15   example in order to increase reaction rate.

Mutant luciferases having increased thermostability are known
from EP-A-524448 and WO/95/25798. The first of these describes
a mutant luciferase having a mutation at position 217 in the
20   Japanese firefly luciferase, in particular by replacing a
threonine residue with an isoleucine residue. The latter
describes mutant luciferases having over 60% similarity to
luciferase from *Photinus pyralis*, *Luciola mingrelica*, *Luciola*
cruciata or *Luciola lateralis* but in which the amino acid
25   residue corresponding to residue 354 of *Photinus pyralis* or 356
of the *Luciola* species is mutated such that it is other than
glutamate, and in particular is other than glutamate,
aspartate, proline or glycine.

30   Co-pending British Patent Application No.9823468.5 and the
International Patent Application derived from it, describes
further such mutants. In this case, proteins are described
which have luciferase activity and at least 60% similarity to
wild-type luciferase such as those from *Photinus pyralis*,
35   *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*
enzyme, but which include mutations at various positions in the
protein, including amongst others, (a) the amino acid residue

corresponding to residue 214 in *Photinus pyralis* luciferase and
to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or
Luciola lateralis luciferase; or

(b) the amino acid residue corresponding to residue 232 in
5   *Photinus pyralis* luciferase and to residue 234 of *Luciola
mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase;
or

(c) amino acid residue corresponding to residue 295 in *Photinus
pyralis* luciferase and to residue 297 of *Luciola mingrelica*,
10   *Luciola cruciata* or *Luciola lateralis* luciferase.

The applicants have found that by mutating (or introducing) an
amino acid at a different position within the luciferase
protein, large shifts in the wavelength of the emitted light
15   may be achieved and/or the enzyme has improved thermostability.
Furthermore, the proton flux of emitted light may be improved,
making the enzyme better suited to *in vivo* assays where glow
kinetics are precluded or *in vitro* assays where CoA or other
'glow kinetic inducing' compounds are not present.
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The present invention provides a recombinant protein having
luciferase activity and at least 60% similarity to a wild-type
luciferase wherein in the sequence of the enzyme, the amino
acid residue corresponding to residue 357 in *Photinus pyralis*
25   luciferase is mutated as compared to the corresponding wild-
type luciferase, such that the luciferase enzyme is able to
emit light at a different wavelength as compared to the
corresponding wild-type luciferase and/or has enhanced
thermostability as compared to the corresponding wild-type
30   luciferase.

Wild-type luciferase sequences which can form the basis of the
recombinant forms of the invention include *Photinus pyralis*,
Luciola mingrelica, *Luciola cruciata* or *Luciola lateralis*,
35   *Hotaria paroula*, *Pyrophorus plagiophthalamus Lampyris
noctiluca*, *Pyrocoelia nayako*, *Photinus pennsylvanica* or

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Phrixothrix (railroad-worms - see Biochem. 38 (1999) 8271-
8279).

Bioluminescent enzymes from species that can use the substrate
D-luciferin (4,5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-
5   thiazole carboxylic acid) to produce light emission may form
the basis of the mutant enzymes of the invention.

Particular wild-type luciferase sequences which can form the
basis of the recombinant forms of the invention include
10   *Photinus pyralis, Luciola mingrelica, Luciola cruciata* or
Luciola lateralis, Hotaria paroula, Pyrophorus plagiophthalamus
Lampyris noctiluca, Pyrocoelia nayako and *Photinus*
pennsylvanica.

15   In particular, the luciferases are enzymes obtainable from
Photinus pyralis, Luciola mingrelica, Luciola cruciata or
Luciola lateralis enzyme. In *Luciola mingrelica, Luciola*
cruciata or *Luciola lateralis* enzymes, the appropriate amino
acid residue is at position 359 in the sequence.
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The sequences of all the various luciferases show that they are
highly conserved having a significant degree of similarity
between them. This means that corresponding regions among the
enzyme sequences are readily determinable by examination of the
25   sequences to detect the most similar regions, although if
necessary commercially available software (e.g. "Bestfit" from
the University of Wisconsin Genetics Computer Group; see
Devereux et al (1984) Nucleic Acid Research 12: 387-395) can be
used in order to determine corresponding regions or particular
30   amino acids between the various sequences. Alternatively or
additionally, corresponding acids can be determined by
reference to L. Ye et al., Biochim. Biophys Acta 1339 (1997)
39-52 which shows the sequences of the enzymes, together with
the numbering, which numbering system is to be used in
35   connection with the present application.

As regards the possible change of the amino acid residue corresponding to residue 357 in *Photinus pyralis* luciferase, most wild-type sequences have an acid residue (aspartic acid or glutamic acid) at this position. The exception to this is some forms of the luciferase of *Photinus pennsylvanica* in which the corresponding residue (355) is the non-polar residue, valine or some forms of *Phrixothrix* luciferase where the corresponding position is V354 in Pv_{GR} or in Ph_{RE}, where it is L354 leucine. Thus in general, the amino acid used as a substitute amino acid at this position is other than aspartic acid, glutamic acid, valine or leucine.

In most cases, therefore, an acidic amino acid residue is replaced with a non-acidic residue, including basic amino acids such as lysine or arginine, non-polar amino acids such as leucine, valine or isoleucine, uncharged polar amino acids such as tyrosine, asparagine, glutamine, phenylalanine, serine, tryptophan or threonine. In particular, it may be replaced with an uncharged polar amino acid such as tyrosine, asparagine, serine or threonine. Particularly preferred amino acid residues for substitution at this position are tyrosine, phenylalanine or tryptophan and most preferably tyrosine. Generally speaking, aromatic residues at this position give rise to the largest shifts and may also assist thermostability.

Where wild-type sequences include non-acidic amino acid residues at this position, they are suitably mutated into different non-acidic residues.

It has been found that by mutating the enzyme in this way, the wavelength of light emitted by the luciferase is shifted, in some cases up to 50nm towards the red end of the spectrum. Thus, D357Y mutant Photinus pyralis luciferase emits light at a wavelength of some 612nm as compared to the wild-type enzyme which emits light at a wavelength of 562nm.

A wavelength shift of 50nm has considerable potential for use in assay applications as a shift of this magnitude can be

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readily defined spectrally. Different coloured luciferases
could be employed as reporter molecules in gene expression
studies, enabling the simultaneous monitoring of more than one
gene, for example as described in WO 95/18853. Multiple
5   analyte testing could also be performed with luciferase as
labels.

The fact that the light in this case is a deep red in colour is
particularly useful in assay methodology. A red mutant could
10   be useful when analysing a solution for ATP which contained
pigments or other compounds which may absorb shorter
wavelengths of light. For example, a red coloured solution
would not absorb red light. Examples of red coloured solutions
which are frequently the subject of such analysis include blood
15   samples or a solution of eukaryotic cell culture medium which
may contain a red coloured pH indicator.

When using a mixture of colourimetric agents such as
luciferases, the ability to generate a deep red signal may be
20   helpful, particularly where another agent in the sample
generates a green signal. A photomultiplier tube used in
photocathode spectral analysis can be set to detect either one
or both peaks generated in a single sample. In other words, it
is possible to distinguish between photon flux from a red and
25   green emitter in the same sample.

Furthermore, it has been found that the wavelength shift can be
affected by the presence of the cofactor coenzyme A (CoA).
This feature gives rise to the possibility that this enzyme
30   could be used in an assay for the cofactor.

As described below, the effect the cofactor coenzyme A on the
in vitro spectrum of emitted light was investigated. As the
concentration of coenzyme A increases the spectral distribution
35   alters and at the highest concentrations of CoA the spectrum is
dominated by wavelengths in the region 590-630nm with a
pronounced peak at 610nm.

Thus in accordance with a further aspect of the invention,
there is provided an assay for determining the presence in a
sample of CoA, which assay comprises adding to a sample
suspected of containing CoA, luciferase as described above

5   together with other reagents which are required to bring about
a luciferase/luciferin reaction, measuring the wavelength of
light emitted from the sample and relating this to the presence
or absence of CoA.

10   Such an assay may be useful in the detection of the state of
growth or activity of cells, for example microorganisms or
eukaryotic cells.

For example, the concentration of CoA in *E. coli* cells is

15   relatively high, and varies considerably with metabolic status.
The mutant enzymes of the invention can be used to monitor the
metabolic status of an organism, particularly the *in vivo*
concentration of the CoA, since the wavelength of the emission
varies depending upon the CoA concentration. Such assays may

20   be particularly useful in situations where CoA is an important
primary metabolite in the production of antibiotics (e.g. in
streptomycetes). Cellular CoA concentrations are also an
important indicator of fatty acid biosynthesis and vary with
the starvation status of the cell. A number of metabolic

25   disorders such as carcinogenesis and diabetes, show
abnormalities in the fatty acid metabolites and consequently
unusual CoA levels. Assays of the invention may be used in the
diagnosis of such conditions. For example, the CoA levels from
within a cell sample, such as a blood sample, from a patient,

30   may be determined by measuring the wavelength of light emitted
from a luciferase of the invention, used in the assay. This
result may be compared with that obtained from a sample of
healthy cells to determine whether the wavelength has changed
and thus that a modified CoA level is present. This may be

35   indicative of a disease state in the patient. Cells are
suitably lysed prior to assay using a known lytic agent.

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It is believed that the amino acid residue at position 357 is
critically associated with the binding site of coenzyme A.
When the surface of the luciferase enzyme was contoured (using
SYBL protein modelling software, Tripos Ltd.) to a resolution

5   of 1 Angstrom (Å), a small polar pocket was noted. This pocket
appears to be lined by residues H310, E354 and D357 and
measured between 8-10Å. When viewed from the top of the
molecule, this pocket appears as part of a larger pocket, lined
by residues H310, E354, D357 and I232. Residues H310 and E354

10   appear to form a bridge across the cleft giving the appearance
of two smaller pockets (See Figure 8).

Without being bound by theory, it seems possible that the
bridging residues may be flexible enough to disengage when the

15   enzyme is in solution to provide a larger pocket (~12Å deep and
~8Å wide) which allows CoA binding. This is consistent with
the energy calculations.

When *E. coli* cells expressing mutants of firefly luciferase of

20   the invention were grown on different carbon sources changes in
the *in vivo* spectrum of emitted light were observed. Switching
from a rich medium (LB) to a defined minimal medium with either
acetate or glucose as the sole carbon source resulted in shifts
to longer wavelengths of emitted light and a reduction in the

25   contribution from shorter wavelengths. This may provide yet a
further means of controlling the wavelength of light emitted
for assay purposes.

Mutation of the 357 position of in the protein has been found

30   to result in enhanced thermostability.

The proteins may contain further mutations in the sequence
provided the luciferase activity of the protein is not unduly
compromised. The mutations suitably enhance the properties of

35   the enzyme or better suit it for the intended purpose in some
way. This may mean that they result in enhanced
thermostability and/or colour shift properties, and/or the K_m
for ATP of the enzymes. Examples of mutations which give rise

to colour shifts are described in WO95/18853. Mutations which
affect K_m values are described for example in WO 96/22376 and
International Patent Application No. PCT/GB98/01026.

5   In general, effects of mutations have been found to be additive
in terms of alterations in the properties.

The mutant luciferases of the invention may include other
specific mutations which enhance thermostability as compared to
10   wild-type luciferase. In particular, at least one of
(a) the amino acid residue corresponding to amino acid 354 of
the *Photinus pyralis* luciferase (356 in *Luciola* luciferase) is
mutated;
(b) the amino acid residue corresponding to position 215 in
15   *Photinus pyralis* luciferase or (217 in *Luciola* luciferase) is a
different hydrophobic amino acid; or
(c) the amino acid residue corresponding to residue 214 in
Photinus pyralis luciferase or to residue 216 of *Luciola*
mingrelica, *Luciola cruciata* or *Luciola lateralis* luciferase;
20   (d) the amino acid residue corresponding to residue 232 in
Photinus pyralis luciferase or to residue 234 of *Luciola*
mingrelica, *Luciola cruciata* or *Luciola lateralis* luciferase;
(e) amino acid residue corresponding to residue 295 in *Photinus*
pyralis luciferase or to residue 297 of *Luciola mingrelica*,
25   *Luciola cruciata* or *Luciola lateralis* luciferase;
(f) amino acid residue corresponding to amino acid 14 of the
Photinus pyralis luciferase or to residue 16 of *Luciola*
mingrelica, or 17 in *Luciola cruciata* or *Luciola lateralis*;
(g) amino acid residue corresponding to amino acid 35 of the
30   *Photinus pyralis* luciferase or to residue 37 of *Luciola*
mingrelica, or to residue 38 of *Luciola cruciata* or *Luciola*
lateralis;
(h) amino acid residue corresponding to amino acid residue 105
of the *Photinus pyralis* luciferase or to residue 106 of *Luciola*
35   *mingrelica*, 107 of *Luciola cruciata* or *Luciola lateralis* or 108
of *Luciola lateralis* gene;

(i) amino acid residue corresponding to amino acid residue 234 of the *Photinus pyralis* luciferase or to residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

(j) amino acid residue corresponding to amino acid residue 420 of the *Photinus pyralis* luciferase or to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

(k) amino acid residue corresponding to amino acid residue 310 of the *Photinus pyralis* luciferase or to residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*;

is different to the amino acid which appears in the corresponding wild type sequence and wherein the luciferase enzyme has increased thermostability as compared to an enzyme having the amino acid of the corresponding wild-type luciferase at this position.

Thus preferred examples of proteins of the invention are mutated wild-type luciferases where more than one amino acid, for example up to 100 amino acid residues, preferably no more than 40 amino acids, and more preferably up to 30 amino acids, are different to the amino acid at the corresponding position in the appropriate wild-type enzyme.

Thus, in one preferred embodiment, the protein of the invention comprises luciferase of *Photinus pyralis*, wherein, in addition to the mutation at the 357 position as described above, at least one of;

a) the amino acid residue corresponding to amino acid 354 of the *Photinus pyralis* luciferase is other than glutamate;

(b) the amino acid residue corresponding to position 215 in *Photinus pyralis* luciferase or is a hydrophobic amino acid other than alanine;

(c) the amino acid residue corresponding to residue 214 in *Photinus pyralis* luciferase is other than threonine;

(d) the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase is other than isoleucine;

(e) amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase is other than phenylalanine;

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(f) amino acid residue corresponding to amino acid 14 of the
Photinus pyralis luciferase is other than phenylalanine;
(g) amino acid residue corresponding to amino acid 35 of the
Photinus pyralis luciferase is other than leucine;
5   (h) amino acid residue corresponding to amino acid residue 105
of the *Photinus pyralis* luciferase is other than alanine;
(i) amino acid residue corresponding to amino acid residue 234
of the *Photinus pyralis* luciferase is other than aspartic acid;
(j) amino acid residue corresponding to amino acid residue 420
10   of the *Photinus pyralis* luciferase is other than serine;
(k) amino acid residue corresponding to amino acid residue 310
of the *Photinus pyralis* luciferase is other than histidine.

Alternatively, the protein of the invention comprises protein
15   the luciferase sequence of *Luciola mingrelica*, *Luciola cruciata*
or *Luciola lateralis* enzyme, and wherein, in addition to the
mutation at position 359 as described above, at least one of
a) the amino acid residue corresponding to amino acid 356 of
the *Photinus pyralis* luciferase is other than glutamate;
20   (b) the amino acid residue corresponding to position 215 in
Photinus pyralis luciferase or is a hydrophobic amino acid
other than alanine or threonine;
(c) the amino acid residue corresponding to residue 216 of
Luciola mingrelica, *Luciola cruciata* or *Luciola lateralis*
25   luciferase is other than glycine (for *Luciola mingrelica* based
sequences) or aparagine (for *Luciola cruciata* or *Luciola
lateralis*) based sequences;
(d) the amino acid residue corresponding to residue 234 of
Luciola mingrelica, *Luciola cruciata* or *Luciola lateralis*
30   luciferase is other than serine;
(e) amino acid residue corresponding to residue 297 of *Luciola
mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is
other than leucine;
(f) amino acid residue corresponding to amino acid 16 of
35   *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is
other than phenylalanine;

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(g) amino acid residue corresponding to residue 37 of *Luciola mingrelica*, or 38 in *Luciola cruciata* or *Luciola lateralis* is other than lysine;

(h) amino acid residue corresponding to amino acid residue 106 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than glycine;

(i) amino acid residue corresponding to amino acid residue 236 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than glycine;

(j) amino acid residue corresponding to residue 422 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine;

(k) amino acid residue corresponding to amino acid residue 312 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* is other than threonine (for *Luciola mingrelica* based sequences) or valine (for *Luciola cruciata* or *Luciola lateralis*) based sequences.

The particular substituted amino acids in any case which give rise to enhanced thermostability can be determined by routine methods as illustrated hereinafter. In each case, different substitutions may result in enhanced thermostability. Substitution may be effected by site-directed mutagenesis of DNA encoding native or suitable mutant proteins as would be understood by the skilled person. The invention in this case is associated with the identification of the positions which are associated with thermostability.

In general however, it may be desirable to consider substituting an amino acid of different properties for the wild type amino acid. Thus hydrophilic amino acid residues may, in some cases be preferably substituted with hydrophobic amino acid residues and vice versa. Similarly, acidic amino acid residues may be substituted with basic residues.

For instance, the protein may comprise a protein having luciferase activity and at least 60% similarity to luciferase

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from *Photinus pyralis*, *Luciola mingrelica*, *Luciola cruciata* or
Luciola lateralis enzyme wherein in the sequence of the enzyme,
at least one of;

(a) the amino acid residue corresponding to residue 214 in
5 *Photinus pyralis* luciferase and to residue 216 of *Luciola*
mingrelica, *Luciola cruciata* or *Luciola lateralis* luciferase is
mutated and is other than threonine in the case of *Photinus*
pyralis luciferase; or

(b) the amino acid residue corresponding to residue 232 in
10 *Photinus pyralis* luciferase and to residue 234 of *Luciola*
mingrelica, *Luciola cruciata* or *Luciola lateralis* luciferase is
mutated and is other than isoleucine in the case of *Photinus*
pyralis luciferase; or

(c) amino acid residue corresponding to residue 295 in *Photinus*
15 *pyralis* luciferase and to residue 297 of *Luciola mingrelica*,
Luciola cruciata or *Luciola lateralis* luciferase is mutated and
is for example, other than phenylalanine in the case of
Photinus pyralis luciferase;
and the luciferase enzyme has increased thermostability as
20 compared to the wild-type luciferase.

The sequences of all the various luciferases show that they are
highly conserved having a significant degree of similarity
between them. This means that corresponding regions among the
25 enzyme sequences are readily determinable by examination of the
sequences to detect the most similar regions, although if
necessary commercially available software (e.g. "Bestfit" from
the University of Wisconsin Genetics Computer Group; see
Devereux et al (1984) Nucleic Acid Research 12: 387-395) can be
30 used in order to determine corresponding regions or particular
amino acids between the various sequences. Alternatively or
additionally, corresponding acids can be determined by
reference to L. Ye et al., Biochim. Biophys Acta 1339 (1997)
39-52.
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With respect to the possible change of the amino acid residue
corresponding to residue 214 in *Photinus pyralis* luciferase,

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the polar amino acid threonine is suitably replaced with a non polar amino acid such as alanine, glycine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine. A particularly preferred substitution for the threonine residue corresponding to residue 214 in *Photinus pyralis* is alanine. A more preferred substitution is cysteine. However, different polar residues such as asparagine at this position may also enhance the thermostability of the corresponding enzyme having threonine at this position.

Other amino acids which appear at this position in wild-type luciferase enzymes include glycine (*Luciola mingrelica, Hotaria paroula*), asparagine (*Pyrophorus plagiophthalamus, GR, YG, YE and OR, Luciola cruciata, Luciola lateralis, Lampyris noctiluca, Pyrocelia nayako Photinus pennsylvanica LY, KW, J19*) and serine (*Phrixothix*). These may advantageously be substituted with non-polar or different non-polar side chains such as alanine and cysteine.

As regards the possible change of the amino acid residue corresponding to residue 232 in *Photinus pyralis* luciferase, the nonpolar amino acid isoleucine is suitably replaced with a different non polar amino acid such as alanine, glycine, valine, lecine, proline, phenylalanine, methionine, tryptophan or cysteine. Other amino acids appearing at this position in wild type sequences include serine and asparagine. Suitably, these polar residues are substituted by non-polar residues such as those outlined above. A particularly preferred substitution for the residue corresponding to residue 232 in *Photinus pyralis* luciferase and to residue 234 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase at group is alanine.

Changes of the amino acid residue corresponding to residue 295 in *Photinus pyralis* luciferase and to residue 297 of *Luciola mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase, may also affect the thermostability of the protein. (This corresponds to position 292 in *Phrixothix* luciferase.) In

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general, the amino acid at this position is a non-polar amino acid phenylalanine or leucine. These are suitably changed for different non-polar amino acids. For example, in *Photinus pyralis*, the non-polar amino acid phenylalanine is suitably replaced with a different non polar amino acid, such as alanine, leucine, glycine, valine, isoleucine, proline, methionine, tryptophan or cysteine. A particularly preferred substitution for the phenylalanine residue corresponding to residue 214 in *Photinus pyralis* luciferase is leucine.

Mutation at the amino acid residue corresponding to amino acid 14 of the *Photinus pyralis* luciferase or to amino acid 16 in Luciola luciferase (13 in *Phrixothrix* luciferase) is also possible. This amino acid residue (which is usually phenylalanine, but may also be leucine, serine, arginine or in some instances tyrosine) is suitably changed to a different amino acid, in particular to a different nonpolar amino acid such as alanine, valine, leucine, isoleucine, proline, methionine or tryptophan, preferably alanine.

Mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase or to amino acid residue 37 in Luciola mingrelica luciferase (38 in other *Luciola* spp.) may also be effective. This amino acid varies amongst wild type enzymes, which may include leucine (*Photinus pyralis*) but also lysine, histidine, glycine, alanine, glutamine and aspartic acid at this position. Suitably the amino residue at this position is substituted with a non-polar amino acid residue or a different non-polar amino acid such as alanine, valine, phenylalanine, isoleucine, proline, methionine or tryptophan. A preferred amino acid at this position is alanine, where this is different to the wild-type enzyme.

Mutations at the amino acid corresponding to position 14 of the *Photinus pyralis* sequence and/or mutation at the amino acid residue corresponding to amino acid 35 of the *Photinus pyralis* luciferase are preferably not the only mutation in the enzyme.

They are suitably accompanied by others of the mutations
defined above, in particular those at positions corresponding
to positions 214, 395 or 232 of *Photinus pyralis* luciferase.

5   Changes of the amino acid residue corresponding to residue 105
in *Photinus pyralis* luciferase and to residue 106 of *Luciola
mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase,
(102 in *Phrixothrix*) may also affect the thermostability of the
protein. In general, the amino acid at this position is a non-

10 polar amino acid alanine or glycine, or serine in *Phrixothrix*.
These are suitably changed for different non-polar amino acids.
For example, in *Photinus pyralis*, the non-polar amino acid
alanine is suitably replaced with a different non polar amino
acid, such as phenylalanine, leucine, glycine, valine,

15 isoleucine, proline, methionine or tryptophan. A particularly
preferred substitution for the alanine residue corresponding to
residue 105 in *Photinus pyralis* luciferase is valine.

Changes of the amino acid residue corresponding to residue 234

20 in *Photinus pyralis* luciferase and to residue 236 of *Luciola
mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase
(231 in *Phrixothrix*), may also affect the thermostability of
the protein. In general, the amino acid at this position is
aspartic acid or glycine and in some cases, glutamine or

25 threonine. These are suitably changed for non-polar or
different non-polar amino acids as appropriate. For example,
in *Photinus pyralis*, the amino acid residue is aspartic acid is
suitably replaced with a non polar amino acid, such as alanine,
leucine, glycine, valine, isoleucine, proline, methionine or

30 tryptophan. A particularly preferred substitution for the
phenylalanine residue corresponding to residue 234 in *Photinus
pyralis* luciferase is glycine. Where a non-polar amino acid
residue such as glycine is present at this position (for
example in *Luciola* luciferase), this may be substituted with a

35 different non-polar amino acid.

Changes of the amino acid residue corresponding to residue 420
in *Photinus pyralis* luciferase and to residue 422 of *Luciola
mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase
(417 in *Phrixothrix* green and 418 in *Phrixothrix* red), may also

5   affect the thermostability of the protein. In general, the
amino acid at this position is an uncharged polar amino acid
serine or threonine or glycine. These are suitably changed for
different uncharged polar amino acids. For example, in
Photinus pyralis, the serine may be replaced with asparagine,

10 glutamine, threonine or tyrosine, and in particular threonine.

Changes of the amino acid residue corresponding to residue 310
in *Photinus pyralis* luciferase and to residue 312 of *Luciola
mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase,

15 may also affect the thermostability of the protein. The amino
acid residue at this position varies amongst the known
luciferase proteins, being histidine in *Photinus pyralis*,
Pyrocelia nayako, *Lampyris noctiluca* and some forms of *Photinus
pennsylanvanica* luciferase, threonine in *Luciola mingrelica*,

20 *Hotaria paroula* and *Phrixothix* (where it is amino acid 307)
luciferase, valine in *Luciola cruciata* and *Luciola lateralis*,
and asparagine in some *Pyrophorus plagiophthalamus* luciferase.
Thus, in general, the amino acid at this position is
hydrophilic amino acid which may be changed for a different

25 amino acid residue which increases thermostability of the
enzyme. A particularly preferred substitution for the histidine
residue corresponding to residue 310 in *Photinus pyralis*
luciferase is arginine.

30 Other mutations may also be present in the enzyme. For
example, in a preferred embodiment, the protein also has the
amino acid at position corresponding to amino acid 354 of the
Photinus pyralis luciferase (356 in *Luciola* luciferase) changed
from glutamate, in particular to an amino acid other than

35 glycine, proline or aspartic acid. Suitably, the amino acid at
this position is tryptophan, valine, leucine, isoleucine are
asparagine, but most preferably is lysine or arginine. This

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mutation is described in WO 95/25798. It has been found that
hydrophobic residues at this position enhance the wavelength
shift of the enzyme, Furthermore, the presence of a large
hydrophobic (V or I), polar (N) or positively charged (K or R)
5   amino acid at position 354 enhances thermostability.

In an alternative preferred embodiment, the protein also has
the amino acid at the position corresponding to amino acid 217
in Luciola luciferase (215 in *Photinus pyralis*) changed to a
10   hydrophobic amino acid in particular to isoleucine, leucine or
valine as described in EP-A-052448.

Proteins of the invention include both wild-type and
recombinant luciferase enzymes. They have at least 60%
15   similarity to wild sequences such as those of *Photinus pyralis*,
Luciola mingrelica, *Luciola cruciata* or *Luciola lateralis*
enzyme in the sense that at least 60% of the amino acids
present in the wild-type enzymes are present in the proteins of
the invention. Such proteins can have a greater degree of
20   similarity, in particular at least 70%, more preferably at
least 80% and most preferably at least 90% to the wild-type
enzymes listed above. Similar proteins are of this type
include allelic variants, proteins from other insect species as
well as recombinantly produced enzymes. They can be readily
25   identified in that they are encoded by nucleic acids which
hybridise with sequences which encode wild-type enzymes under
stringent hybridisation conditions. Such conditions would be
well understood by the person skilled in the art, and are
exemplified for example in Sambrook et al. (1989) Molecular
30   Cloning, Cold Spring Harbor Laboratory Press). In general
terms, low stringency conditions can be defined as 3 x SCC at
about ambient temperature to about 65°C, and high stringency
conditions as 0.1 x SSC at about 65°C. SSC is the name of a
buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is
35   three times as strong as SSC and so on.

In particular, the similarity of a particular sequence to the
sequences of the invention may be assessed using the multiple

alignment method described by Lipman and Pearson, (Lipman, D.J.
& Pearson, W.R. (1985) Rapid and Sensitive Protein Similarity
Searches, Science, vol 227, pp1435-1441). The "optimised"
percentage score should be calculated with the following
5   parameters for the Lipman-Pearson algorithm:ktup =1, gap
penalty =4 and gap penalty length =12. The sequences for which
similarity is to be assessed should be used as the "test
sequence" which means that the base sequence for the
comparison, such as the sequence of *Photinus pyralis* or any of
10 the other sequences as recorded in Ye et al., supra., should be
entered first into the algorithm.

Particular examples of proteins of the invention are wild-type
luciferase sequence with one or more of the mutations as
15 outlined above.

The invention further provides nucleic acids which encode the
luciferases as described above. Suitably, the nucleic acids
are based upon wild-type sequences which are well known in the
20 art. Suitable mutation to effect the desired mutation in the
amino acid sequence would be readily apparent, based upon a
knowledge of the genetic code.

In a preferred embodiment of the invention, the nucleic acid is
25 a synthetic gene. Suitably, the synthetic gene is engineered
to remove codons rarely found in highly expressed genes from
common expression hosts such as *E. coli* and, at the same time,
avoid the introduction of codons rarely found in genes coding
for beetle luciferases. This approach ensures that the new gene
30 has a codon utilisation that is optimal for both *E. coli* and
insect expression systems.

For example, wherever possible the codons for the amino acids
arg, leu, ile, gly and pro were changed to CGT or CGC (arg),
35 CTG, CTT or CTC (leu), ATC or ATT (ile), GGT or GGC (gly), and
CCG CCA or CCT (pro), thus eliminating rare codons. In the
case of the synthetic gene illustrated below (SEQ ID NO 1) and
in Figure 14, this resulted in a total of 139 silent mutations

creating 62 new non-rare codons (11% of the total). The first 8 nucleotides shown in Figure 14 form part of the ribosome binding site and thus do not code. The coding sequence begins with the methionine residue indicated by an up arrow. This coding sequence and closely similar sequences, for example sequences which have at least 90% similarity or preferably at least 95% similarity form a preferred aspect of the invention.

Another useful feature which may be employed when producing a synthetic assembly is the incorporation of new unique restriction sites. These sites make mutagenesis, in particular combinatorial cassette mutagenesis, of the gene simpler and more efficient. In particular, it may be desirable to create unique restriction sites within the cDNA coding for subdomain B in the enzyme. Additionally creation of a unique restriction site at the extreme 3' end of the gene to allow simple fusions and/or removal of the peroxisome targeting sequence may be advantageous.

In the example illustrated hereinafter, nine new unique restriction sites were engineered, mostly in the central third of the gene, and a unique Hind III site was generated at the extreme 3' end of the gene to allow for simple C-terminal fusions (Figure 12).

Finally, use of a synthetic gene allows for the introduction of mutations to increase the thermostablilty of the gene product, or to otherwise modify the properties of the product as desired. In the Example illustrated hereinafter for instance, three non-silent mutations were engineered to introduce the thermostabilising amino acid changes T214C, E354K and D357F into the polypeptide.

The nucleic acids of the invention are suitably incorporated into an expression vector such as a plasmid under the control of control elements such as promoters, enhancers, terminators etc. These vectors can then be used to transform a host cell, for example a prokaryotic or eukaryotic cell such as a plant or

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animal cell, but in particular a prokaryotic cell such as *E. coli* so that the cell expresses the desired luciferase enzyme. Culture of the thus transformed cells using conditions which are well known in the art will result in the production of the

5   luciferase enzyme which can then be separated from the culture medium. Where the cells are plant or animal cells, plants or animals may be propagated from said cells. The protein may then be extracted from the plants, or in the case of transgenic animals, the proteins may be recovered from milk. Vectors,

10 transformed cells, transgenic plants and animals and methods of producing enzyme by culturing these cells all form further aspects of the invention.

The *Photinus pyralis* D357Y mutant luciferase was created by

15 random mutagenesis as described hereinafter. It was found that the D357Y single point mutation produces a large colour shift in the wavelength of light emitted and also has greater thermostability than wild type luciferase. Further investigations have revealed that a range of substitutions at

20 this position give rise to good thermostability and/or to large colour shifts.

Particular examples of mutant enzymes of *Photinus pyralis* which fall within the scope of the invention include the following:

25

    D357Y
    D357F
    D357W
    D357K
30 D357N
    D357I
    E354I/D357Y
    E354V/D357Y
    E354C/D357Y
35 E354R/D357Y
    E354S/D357Y
    E354N/D357Y
    E354K/D357M

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E354R/D357L
E354W/D357W
E354H/D357W
E354R/D357F
5   E354K/D357F
E354S/D357F
E354M/D357F
E354A/D357R
E354A/D357F
10   E354T/D357Y
E354A/D357N
I351M/E354R/D357V
E354S/D357V
E354R/D357W
15   E354R/D357M
E354R/D357S
E354N/D357S

or equivalents of any of these when derived from the
20 luciferases of other species.

The mutations for the creation of the above mutants were
introduced to the luciferase gene on plasmid pET23 by site-
directed mutagenesis, (PCR) or combinatorial cassette
25 mutagenesis. The oligonucleotides added to the PCR reaction in
order to effect the relevant mutations are given below.

It has been reported previously that the effect of point
mutations at the 354 and 215 positions are additive. This
30 invention provides the possibility of combining three or more
such mutations to provide high thermostability in a mutant
enzyme which has a large colour shift.

Luciferase proteins of the invention will advantageously be
35 employed in any bioluminescent assay which utilises the
luciferase/luciferin reaction as a signalling means. There are
many such assays known in the literature. The proteins may
therefore be included in kits prepared with a view to

performing such assays, optionally with luciferin and any other
reagents required to perform the particular assay.

The invention will now be particularly described by way of
5   example with reference to the accompanying diagrammatic
drawings in which:

Figure 1 is a log graph showing % remaining activity versus
time of 45°C incubation of several mutant enzymes in accordance
10   with the invention;

Figure 2 shows the spectral peaks obtained by incubating *E.
coli* cells expresing luciferase enzymes in a citrate buffer
with D-luciferin where where the enzyme used is (a) recombinant
15   wild-type *Photinus pyralis* luciferase, (b) a D357K mutant, (c)
a D357N mutant,   (d) a D357W mutant, (e) a D357I mutant, (f) a
D357F mutant, (g) a D357Y mutant and (h) a double mutant E354I
+ D357Y;

20   Figure 3 is a graph showing the % remaining activity versus
time of three mutant enzymes, E354I, D357Y and the double
mutant (DM) E354I/D357Y;

Figure 4 shows the emission spectra of (a) recombinant wild
25   type enzyme and (b) the double mutant (DM) E354I/D357Y;

Figure 5 is a graph showing the rate decay of photon emissions
of recombinant wild-type (♦) r-wt and a D357K mutant enzyme
(¦).
30
Figure 6 shows molecular modelling diagram, illustrating a
potential CoA binding pocket within the luciferase enzyme;

Figure 7 shows the in vivo bioluminescent spectra emitted by E.
35   coli cells expressing mutant *P. pyralis* luciferase D357Y (a)
growth on LB; (b) growth on minimal medium and sodium acetate;
(c) growth on minimal medium and glucose;

24

Figure 8 shows the in vivo bioluminescent spectra emitted by E. coli cells expressing mutant *P. pyralis* luciferase E354K/D357M (a) growth on LB; (b) growth on minimal medium and sodium
5   acetate; (c) growth on minimal medium and glucose;

Figure 9 is a graph showing the effect of CoA on spectral distribution of light emitted by mutant *P. pyralis* luciferase D357Y;
10

Figure 10 is a graph showing the normalised data of the effect of CoA on spectral distribution of light emitted by mutant *P. pyralis* luciferase D357Y;

15   Figure 11 is a graph showing the effect of CoA on spectral distribution of light emitted by mutant *P. pyralis* luciferase E354I/D357Y (Figure 11a) and normalised data (Figure 11b);

Figure 12 illustrates the restriction sites modifications
20   utilised in the construction of a synthetic luciferase gene;

Figure 13 illustrates constructs used in the synthesis of a luciferase gene;

25   Figure 14 shows the cDNA sequence (SEQ ID NO 1) of the synthetic luciferase gene (including nucleotides 1-8 which form part of the ribosome binding site but are not coding) and the encoded amino acid sequence which starts at the methionine residue indicated by the up arrow (SEQ ID NO 2); and
30

Figure 15 illustrates the thermostability of mutants including the mutant encoded by the synthetic gene at 50°C.

25

Example 1
Identification and Characterisation of Mutant Luciferase
Two libraries of firefly (Photinus pyralis) luciferase, created
used error-prone PCR [M. Fromant et al., Anal. Biochem. (1995)
5   224, 347-353], were prepared. One library comprised of error-
prone PCR products of the full length luc gene, cloned into the
T7 expression system pET23a, (Novagen Inc., Madison, WI,
U.S.A.). A second library consisted of the error-prone PCR
products of a short section of the luc gene, covering amino
10   acids 199-352, cloned in the vector pBSK(+), (Stratagene, La
Jolla, CA, U.S.A.).

The pET23a library was expressed in E. coli strain BL21(DE3),
(E. coli B F dcm ompT hsdS($r_B^-m_B^-$) galλ (DE3)).
15

The pBSK(+) library was expressed in HB101 E. coli cells,
(supE44 ara14 galK2 lacY1 Δ(gpt-proA)62 rpsL20 (Str^r) xyl-5 mtl-
1 recA13 Δ(mrcC-mrr) HsdS$^-$ (r$^-$m$^-$). pET23a and pBSK(+) both carry
the gene for β-lactamase and confer ampicillin resistance to E.
20   coli cells harbouring the plasmid.

An E. coli strain was transformed with the prepared library by
electroporation, using a BIORAD E. coli Pulser, and grown
overnight at 37°C on LB agar, containing ampicillin at a
25   concentration of 50µg/ml. The cells were transferred to nylon
membranes, (Osmonics, Minnetonka, Minnesota, U.S.A.), and
sprayed with luciferin solution (500µM D-luciferin, potassium
salt, in 100mM sodium citrate buffer, pH 5.0). The colonies
were viewed using an AlphaImager™ 1200 Documentation and
30   Analysis System (Flowgen, Lichfield, Staffordshire, UK). This
integrated the bioluminescence emitted over a specified period
of time to produce an image of the light emitted by the
colonies. The brightness of luminescence was taken as an
indication of the thermostability of luciferase.
35

26

The colonies were then screened for thermostability. Colonies were selected on the basis of brightness of light emitted and were isolated for further characterisation. In some screens, the *E. coli* colonies were incubated at 42°C for 2 hours prior to

5 screening so that the thermostable mutants could be selected. Colonies isolated from the primary screen were patched onto nylon membranes and also grown overnight in LB medium containing ampicillin. The patches were sprayed with luciferin solution and viewed in the AlphaImager™. This secondary screen

10 helped to positively identify clones for *in vitro* analysis of luciferase activity. *E. coli* clones expressing possible thermostable enzymes were assayed *in vitro* for luciferase activity and thermostability.

15 *In vitro* assays for luciferase activity were performed at room temperature using the Promega Luciferase Assay System (Promega Corporation, Madison, WI, U.S.A.).

The luciferase reaction was initiated by the addition of 10µl

20 crude cell extract to 100µl Promega Luciferase Assay Cocktail (1 in 2 dilution). The resultant bioluminescence was measured using a Biotrace M3 luminometer.

Crude cell extracts were prepared as described in the Promega

25 technical bulletin no. 101. Aliquots of *E. coli* overnight cultures were lysed in cell culture lysis reagent, (25mM Tris-phosphate pH7.8, 2mM dithiothreitol (DTT), 2mM 1,2-diaminocyclohexame-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, 1.25 mg/ml hen lysozyme) for 10 minutes at room

30 temperature. Crude lysate were then stored on ice prior to assay.

The properties of the enzymes were further tested in time-dependent inactivation studies. Eppendorf tubes containing

35 50µl aliquots of crude cell extract were incubated in a water bath at a given temperature. At set time points tubes were

27

removed and cooled on ice prior to assay. Remaining luciferase
activity was expressed as a percentage of the original
activity.

5   Log graphs of percentage remaining activity versus time of
incubation were plotted and used to calculate $t_{1/2}$ values. $T_{1/2}$
is the time taken for the enzyme to lose 50% of its original
activity after incubation at a given temperature. $T_{1/2}$ values,
(time for activity to reduce to 50% of original activity), were

10   determined in crude extracts at 37°C from log graphs of %
remaining activity versus time (not shown).

Plasmid DNA from *E. coli* clones expressing the most
thermostable luciferase as determined above, was sequenced in

15   order to determine the mutations responsible for the
thermostability of the enzyme.

Plasmid DNA was prepared using the QIAGEN QIAprep Spin Miniprep
Kit, (QIAGEN Ltd, Crawley, W. Sussex, UK), following the

20   protocol for using a microcentrifuge (QIAprep Miniprep Handbook
04/98).

All DNA sequencing was undertaken by Babraham Tech[nix],
Cambridge, UK, using an ABI PRISM™ 377 DNA Sequencer and the

25   ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction
Kit (Perkin Elmer Applied Biosystems) which is based upon the
dideoxy chain termination method [F. Sanger et al., Proc. Natl.
Acad. Sci. U.S.A. 74, (1977) 5463-5467].

30   As a result of this work, the novel mutant D357Y was
identified.

The crystal structure of luciferase [E. Conti et al.,
Structure, 4 (1996) 287-298] shows that position 357 is

35   situated on the surface of the protein and is close to position
354, which can affect both thermostability and spectral

28

properties. This indicates that this region could be important
in terms of the thermostability of the enzyme.

D357Y is a particularly thermostable mutant, being the most
5   thermostable luciferase, with a single amino acid change.

Example 2
Site-Directed Mutagenesis to create other 357 mutants
In order to evaluate different mutations at the 357 position,
10   site-directed mutagenesis was performed using the Stratagene
QuikChange™ Site-Directed Mutagenesis Kit, (Stratagene, La
Jolla, CA, U.S.A.). The plasmid pPW601a J54, (PJW, MoD Report,
3/96), was used in all site-directed mutagenesis. All products
of the mutagenesis reactions were transformed into *E. coli*
15   strain XL1-Blue, [*e14⁻(mcrA⁻) Δ(mcrCB-hsdSMR-mrr)171 endA1*
supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 (Kanʳ)
uvrC [F′ proAB lacIqZΔM15 Tn 10 (Tetʳ) Amy Camʳ]]
Oligonucleotide primers were synthesized by Sigma-Genosys Ltd.,
Cambridge, UK and were designed using an intelligent doping
20   system [A.R Arkin et al., Bio-technology, (1992)10, 297-300,
W,. Huang et al., Anal. Biochem. 218, 454-457] were used to
design degenerate oligonucleotide primers to produce groups of
possible mutations rather than using individual primers for
each amino acid substitution.
25

In this way, libraries of amino acid substituted luciferase
mutants were produced.
The following oligonucleotides (and their complementary
partners) were used:

Oligonucleotide Primer (5'→3')	Amino Acid Substitution
cacccgaggggggat[tat]aaaccgggcgcgg (SEQ ID NO 4)	Y
cacccgaggggggat[(gac)(tc)(c)]aaaccgggcgcggtcgg (SEQ ID NO 5)	A,I,L,T,V,P
cacccgaggggggat[(t)(gat)(gc)]aaaccgggcgcggtcgg (SEQ ID NO 6)	C,F,L,W,Y,X
cacccgaggggggat[(ac)(ga)(gc)]aaaccgggcgcggtcgg (SEQ ID NO 7)	R,S,K,N,H,Q

The libraries of mutants were screened as previously for
thermostability. The number of colonies to be screened was
5 calculated using the equation [S. Climie et al., J. Biol. Chem.
265 (1990) 18776-18779]

$$N = [\ln(1-P)]/[\ln((n-1)/n)]$$

Where N is the number of colonies to be screened, n is the
10 number of possible codons at the target position and P is the
probability that every codon in the mixture is sampled for
screening at least once. The calculation was based on P=0.95.
The mutants obtained from site-directed mutagenesis were
assayed for luciferase activity and characterised in time-
15 dependent thermoinactivation studies.

Mutants identified as desirable in this way were grown in 400ml
LB medium, containing ampicillin, to $A_{260} \approx 0.5$. Luciferase
expression was then induced by addition of isopropyl β-
20 thiogalactoside (IPTG) to a final concentration of 1mM. The
cells were then incubated at 30°C, with shaking, for 3 hours
prior to harvesting by centrifugation. The resultant cell
pellet was resuspended in 10ml B-PER™ Protein Extraction
Reagent, (Pierce Chemical Company, Rochford, U.S.A.), 1mM DTT
25 to produce a crude extract, following the B-PER™ protocol for
Maxi-Scale Bacterial Protein Extraction. Reconstituted Sigma
Protease Inhibitor Cocktail, 500µl, (Product No. P8465, Sigma,

Saint Louis, Missouri, U.S.A.), was added to the B-PER™
solution to inhibit endogenous proteases. The cell lysate was
then centrifuged at 30 000g for 30 minutes.

5   The supernatant of the crude extract was subjected to
fractionation with ammonium sulphate. The fraction that
precipitated between 30% and 55% saturation contained
luciferase activity. This material was resuspended in 0.5ml
Tris HCl pH8.0, 1mM DTT and used for thermoinactivation and
10 spectral studies.

The replacements D357L, T, V, W, R, I, S, K, N and F were
introduced. These mutants were characterised in *in vitro*
thermoinactivation studies of crude extracts.
15
The partially purified extracts were diluted, 1 in 11, into a
thermoinactivation buffer: 50mM potassium phosphate buffer
pH7.8 containing 10% saturated ammonium sulphate, 1mM
dithiothreitol and 0.2% BSA.
20
110µl aliquots of protein solution were incubated at 40°C or
45°C for set periods of time and cooled on ice prior to assay.
Luciferase activity was then measured as described in Example
1, using Promega Luciferase Assay Reagent (1 in 2 dilution).
25
The results are shown in Tables 2 & 3 and in Figure 1. T1/2
values were determined in crude extracts at 40°C (Table 2) and
45°C (Table 3).

31

Table 2

Mutant	$T_{1/2}$
D357K	2.2
D357R	4.2
D357S	4.6
D357N	4.8
D357V	5.9
D357T	7.3
D357L	11.3
D357I	18.0
rWT	<1.0

Table 3

Mutant	$T_{1/2}$
D357W	2.5
D357F	6.5
D357Y	10.4
RWT	<1.0

All the substitutions displayed enhanced thermostability in comparison to recombinant wild type.

5

Example 3

Changes in Wavelength of Emitted Light

Amino acid replacements at position 357 were also observed to affect the *in vivo* spectra of light emitted by the enzyme. An

10   aliquot, (250μl), of *E. coli* cell cultures, as described in Example 2 were grown overnight at 37°C, was spun down in a microcentrifuge and the supernatant removed. Cells expressing different mutant luciferases were incubated in a citrate buffer (pH5.0) containing 150μl D-luciferin and the light emitted from

15   the *in vivo* reaction was analysed by measuring the emission spectra using a SPECTRAmax® Microplate Spectrofluorometer, (Molecular Devices Corp. California, U.S.A.). Large changes in the spectral peak as well as the distribution of wavelengths was observed for the mutants D357Y, F and I (Figure 2(a)-(g)).

20   These results are summarised in Table 4 below.

In addition, the *in vivo* luminescence of the mutants was assessed by eye in a dark room. The D357 mutants displayed a variety of colours in their luminescence spectra. In

25   particular, D357Y, F and I showed significant shifts to longer wavelengths of emitted light.

In some cases, (e.g. D357F), the change in colour of light
emission appeared to be due, not only to a shift in λ_{max}, but to
a difference in contributions to the spectra from different
wavelengths of visible light.

Table 4

Mutant	λ_{max} (nm)	Deviation from rWT (nm)
rWT	558	-
D357K	556	-2
D357N	558	0
D357W	558	0
D357I	606	+48
D357F	611	+53
D357Y	613	+55

Recombinant wild type (r-wt) enzyme was used for comparison of
λ_{max} of *in vivo* light emission of some of the 357 mutants.
D357Y, F and I display considerable shifts in their wavelength
maxima.

Example 4
Enzyme properties in the presence or absence of CoA
D357Y was partially purified by ammonium sulphate
precipitation, as described in the Example 1. This partially
purified D357Y enzyme (5µl) was mixed with 150µl Promega
Luciferase Assay Reagent. Another aliquot was mixed with an
equivalent assay buffer in which CoA is absent, (25mM Tris
Tricine pH7.8, 5.0mM MgSO$_4$, 0.1mM EDTA, 2mM DTT, 470µM D-
luciferin, 530µM ATP). The emission spectra of the two
reactions were measured and are shown in Figures 9 and 10.

The spectra display a marked difference in bioluminescent
emission in the absence and presence of CoA, with dramatic
shift in λ_{max}. The effect of CoA on the kinetics of the
luciferase reaction can also be seen by in the difference in
RLU scales. (RLU - Relative Light Units).

33

This difference in emission gives rise to the possibility of using the enzyme in an assay to detect the presence of CoA.

Example 5

5 Preparation and Properties of Double Mutant
Using site-directed mutagensis as described in Example 2, a double mutant of E354I + D357Y was engineered in order to study any cumulative effects upon thermostability and colour of light emission.

10

The partially purified double mutant, E354I + D357Y, was diluted, 1 in 11, into a thermoinactivation buffer: 50mM potassium phosphate buffer pH7.8 containing 10% saturated ammonium sulphate, 1mM dithiothreitol and 0.2% BSA.

15

110μl aliquots of protein solution were incubated at 45°C for set periods of time and cooled on ice prior to assay. Luciferase activity was then measured as previously, using Promega Luciferase Assay Reagent (1 in 2 dilution).

20

The double mutant displayed a marked increase in thermostability in comparison to the single mutants E354I and D357Y individually, (see Figure 3). Thermoinactivation studies of partially purified double mutant confirmed the increased
25 thermostability of the mutant, giving a $t_{1/2}$ value of 7.7 min when inactivated at 45°C.

It was noted that the double mutant displays a much deeper red colour of luminescence than the individual mutants of E354I and
30 D357Y, displaying additivity of colour of luminescence.

The emission spectra of recombinant wild type and the crude extract of the double mutant E354I + D357Y were also measured using the assay buffer described in Example 3.

35

Emission spectra measured *in vivo* give a λ_{max} of 611nm. However, the spectrum has a greater contribution of

34

luminescence from the red region of wavelengths, leading to its
deeper red appearance when visualised by eye. Emission spectra
in crude extracts displayed a definite change in spectral shape
and a wavelength shift of 44nm, relative to rWT, (see Figure

5 4).

The *in vivo* emission spectrum of the double mutant shows both a
sharpening of the bandwidth for the peak wavelength of emitted
light (613nm) and a decrease in the contribution from

10 wavelengths of light in the region 540-560nm.

The dramatic effect of these mutations indicates the importance
of this region of the enzyme to the colour of bioluminescent
light.

15

Example 6
Improved Photon Flux
The *in vivo* bioluminescence of *E. coli* cells expressing the
mutant D357K was observed to be very bright relative to the

20 other mutants at this position. The flash kinetics of this
enzyme was analysed using a luminometer, which could measure
the rate of photon emission over time. Aliquots of *E. coli*
cell free extracts containing recombinant wild type enzyme or
the mutant D357 were added to a luciferase assay cocktail,

25 which did not contain any reagents that would promote glow
kinetics e.g. coenzymeA. The rate of decay of photon emission
was measured over time (15s) for both enzymes was observed to
be significantly slower for the mutant D357K (Figure 4). In
other words the mutant enzyme has reaction kinetics, which are

30 inhibited to a lesser degree, over at least the first 15
seconds of the reaction, than the recombinant wild type enzyme.

Example 7
Combinatorial cassette mutagenesis at positions E354 and D357
35 Step 1
Engineering plasmid pPW601aJ54 for cassette mutagenesis
Two new unique restriction sites were introduced into the *luc*
gene, in the plasmid pPW601a/J54, using two pairs of synthetic

oligonucleotides (see below). A total of six silent mutations
introduced a SpeI and a KpnI restriction site, 63 base pairs
apart, within the gene. Plasmid containing these new sites was
called pPW601aJ54SpeI/KpnI. The presence and proximity of these
5   restriction sites makes it possible to use combinatorial
cassette mutagenesis to explore the effects of random
substitutions at amino acid positions 354 and 357 in the
primary sequence of firefly luciferase.

10   SpeI (a) 5'-gggctcactgagactacTAGTgctattatgattacacccg-3'
nt1021- nt1060   (SEQ ID NO 8)

SpeI (b) 5'-cgggtgtaatcagaatagcACTAgtagtctcagtgagccc-3'
(SEQ ID NO 9)
15

KpnI (a) 5'-ggcgcggtcggtaaagtGgtAccattttttgaagcg-3'   nt1078-
nt1113   (SEQ ID NO 10)

KpnI (b) 5'-cgcttcaaaaaatggTacCactttaccgaccgcgcc-3'
20   (SEQ ID NO 11)
Nucleotides highlighted in bold form the endonuclease
recognition site and those in upper case the position of the
point mutations necessary to create the site.

25   Step 2
Cassette design and library construction
A pair of synthetic oligonucleotides was synthesised which when
annealed created a double stranded cassette which could be
ligated directly into plasmid pPW601aJ54SpeI/KpnI digested at
30   the new restriction sites. The cassette was designed to
introduce all possible combinations of the 20 naturally
occurring amino acids at positions 354 and 357 in the primary
sequence.

35   Looplib2A   5'-
ctagtgctattctgattacacccNNG/CggggatNNG/Caaaccgggcgcggtcggtaaagtg
gta-3'   (SEQ ID NO 12)

36

Looplib2B   5'-
cactttaccgaccgcgcccggtttG/CNNatccccG/CNNgggtgtaatcagaatagca-3'
(SEQ ID NO 13)

2μg of each of the loop library oligonucleotides was mixed in a
5   buffer containing 50mM Tris-HCl pH 7.4, 25mM NaCl, and heated
to 100°C for 3min. This solution was then cooled slowly in a
heating block to <50°C to anneal the complimentary sequences.
The annealed oligonucleotides were then ligated into plasmid
pPW601aJ54SpeI/KpnI, which had been digested with SpeI and
10   KpnI. Aliquots of the ligation reaction were then used to
transform *E. coli* HB101 cells using electroporation. After
electroporation transformed cells were plated out on LB agar
plates containing 50μg/ml ampicillin and grown overnight at
37°C. The following day 869 colonies were picked at random from
15   the plates and used to inoculate 1ml of LB containing
ampicillin in 96 square-well plates (Beckman). The plates were
covered and the cells grown overnight at 37°C with shaking.

Step 3
20   *In vivo* screening the randomly selected clones
The next morning 50μl aliquots of the stationary phase
overnight cultures were transferred to two clear plastic round
bottom 96 well microtitre plates (Dynex). One plate was covered
and incubated on a heated block for 8 minutes (block surface
25   temperature 45°C) whilst the other was kept at 37°C. The *in vivo*
luciferase activity in the cells from both plates was then
detected and recorded, at room temperature, by adding 50μl of a
100mM sodium citrate buffer pH 5.0 containing 0.5mM D-luciferin
to the wells and then transferring the plate to a video camera
30   imager capture system (Alpha Imager). The light emitted by the
heated and control cultures was integrated over 1 or 2 minutes
and the image recorded on thermal paper film.

Seventy-nine cultures exhibiting the greatest bioluminescence,
35   as determined by the brightness of the image recorded on film,
were selected for a second round of screening. This time the

cultures were incubated for 16 minutes on the heating block
prior to being assayed. Of the 55 clones selected from the *in
vivo* thermostability screens 25 were chosen for *in vivo*
spectral analysis. These clones were grown overnight in LB at

5   37°C and the next morning 200ul of the overnight cultures was
centrifuged and the *E. coli* cell pellets were resuspended in
150µl of 100mM sodium citrate buffer pH 5.0 containing 0.5mM D-
luciferin. The resuspended cells were then placed in a white
plastic microtitre plate and the *in vivo* bioluminescent

10   emission spectrum emitted by each of the mutant luciferases was
analysed using a Molecular Devices Spectramax 96 well plate
fluorimeter. The results are summarised in the Table 1 below.

Step 4

15   Identification of mutations
Plasmid DNA was prepared from the 25 clones selected by *in vivo*
screening and sequenced using gene specific sequencing primers.
Mutations resulting in amino acid changes at positions 354 and
357 in the primary sequence were identified. One mutant, also

20   contained an additional mutation resulting in an amino acid
substitution at position I351 (Table 5).

Table 5

Mutant Enzyme	Mutations	Peak Wavelengths (nm)
1	E354V/D357Y	614
2	E354I/D357Y	612
3	E354C/D357Y	612
4	E354R/D357Y	600
5	E354S/D357Y	612
6	E354N/D357Y	608
7	E354K/D357M	556,606
8	E354R/D357L	588
9	E354W/D357W	610
10	E354H/D357W	606
11	E354R/D357F	596
12	E354K/D357F	608

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Mutant Enzyme	Mutations	Peak Wavelengths (nm)
13	E354S/D357F	610
14	E354M/D357F	610
15	E354A/D357R	556
16	E354A/D357F	610
17	E354T/D357Y	612
18	E354A/D357N	560
19	I351M/E354R/D357V	606
20	E354S/D357V	556,608
21	E354R/D357W	600
22	E354R/D357M	596
23	E354R/D357S	592
24	E354N/D357S	600
rWT	E354/D357	552

where rWT signifies recombinant wild-type.

A number of mutant luciferases were selected from the *in vivo*
assays for thermostability. The majority of these luciferases
also show large changes in the *in vivo* spectrum of emitted
light with many showing greater contributions from longer
wavelengths of light (>580nm). A number of spectra also showed
a significant narrowing of bandwidth around a single peak of
610-614nm.

Replacements of E354 and D357 with a hydrophobic and an
aromatic amino acid respectively e.g. E354V, D357Y results in
the largest change in the in vivo spectrum which shows a single
peak, of narrow bandwidth, around 612nm.

Example 8
In vitro screening for thermostability
Cell free extracts of the selected clones were prepared by
lysis and the thermostability of the luciferase from each
extract was determined in a thermal inactivation experiment.
50µl of each extract was placed in an eppendorf tube and

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incubated in a waterbath heated to 45°C for 4, 9 and 16 minutes.
At the appropriate timepoint the aliquot was removed and the
remaining luciferase activity measured. Table 6 shows the
percent remaining activity versus time for all mutant enzymes
as well as recombinant wild type.

Table 6

Enzyme No.(see Table 5)	Percentage activity remaining after incubation at 45°C			
	0 min	4 min	9 min	16 min
1	100	95	87	75.4
2	100	99	84.7	67.7
3	100	92	73	53.3
4	100	94	89	71.4
5	100	85	72.2	53
6	100	93	84.8	71
7	100	63.7	31	11.7
8	100	58.6	19	4.9
9	100	85.4	65.3	42.3
10	100	65.5	27.8	10.6
11	100	88.6	70	54
12	100	90	69	52
13	100	83	60.5	39
14	100	80	61	39
15	100	1.7	0.1	nd
16	100	90	76	63
17	100	91	78	60
18	100	19	1.8	nd
19	100	17	1.4	nd
20	100	17	1.1	nd
21	100	71	63	34
22	100	80	40	21
23	100	29	4	0.6
24	100	28	4	0.4
25 (D357K)	100	0.1	nd	nd
rWT	100	0.05	nd	nd

where "nd" indicates not done.

The results indicate that the most thermostable luciferases were those with an aromatic amino acid at position 357 (Y, F or W) and a large hydrophobic (V or I), polar (N) or positively charged (K or R) amino acid at position 354.

Example 9
Effect of growth conditions on the in vivo spectrum of emitted light.
The effect of different carbon sources on the spectrum of emitted light from *E. coli* BL21(DE3) cells expressing mutant luciferases D357Y or E354K + D357M (7 above) was investigated.

A 50ml culture of cells was grown to mid log phase on LB medium and then harvested by centrifugation. The cell pellet was resuspended in 1ml of sterile distilled water and a 100ul aliquot of this suspension was then used to inoculate 5ml of fresh LB, M9 minimal medium + 2mM sodium acetate or M9 minimal medium + 2mM glucose in a 25ml Sterilin tube. The cultures were allowed to continue growing, at 37°C with shaking, and after 90 minutes (D357Y) or 120 minutes (enzyme 7) a 200µl aliquot of cells was removed centrifuged and resuspended in 150ul of 100mM sodium citrate buffer pH 5.0 containing 0.5mM D-luciferin. The resuspended cells were then placed in a microtitre plate and the in vivo bioluminescent emission spectrum emitted by each of the mutant luciferases was analysed using a Molecular Devices Spectramax 96 well plate fluorimeter. The results are shown in Figures 7 and 8.

The results show that switching from a rich medium (LB) (Figure 7a, 8a) to a defined minimal medium with either acetate (Figure 7b, 8b) or glucose (Figure 7c, 8c) as the sole carbon source resulted in shifts to longer wavelengths of emitted light and a reduction in the contribution from shorter wavelengths.

Example 10

Purification and spectral characterisation of recombinant wild
type, and mutant luciferases

Recombinant wild type *Photinus pyralis* enzyme and the mutant
luciferases D357Y and E354I + D357Y were purified to
homogeneity in order to analyse the effect of the cofactor
coenzyme A' on the spectrum of the bioluminescent reaction. All
three luciferases were purified as fusions to a 143 amino acid
carbohydrate binding module (CBM) from the anaerobic fungus
Piromyces equii. This CBM has been shown to bind selectively to
acid swollen cellulose and the soluble carbohydrates
galactomannan and glucomannan, forming the basis for a simple
single step affinity purification scheme.

Luciferases fused to the CBM can be bound to cellulose in crude
cell free extracts, washed, and then eluted selectively using
soluble polysaccharides. Fusion proteins purified this way were
used in assays to measure the wavelengths of emitted light in
reactions containing different amounts of coenzyme A. Enzyme
(5µl) was added to 100µl of assay reagent, 25mM Tris-Tricine pH
7.8, 5.0mM MgSO4, 0.1mM EDTA, 530µM ATP and 470µM D-luciferin,
containing different amounts of coenzyme A. Figures 9-11 show
the effect of increasing concentrations of coenzyme A on the
spectrum of light emitted by the purified luciferases D357 and
E354I + D357Y.

In vivo assays of the spectrum of bioluminescent light emitted
by *E. coli* cells expressing firefly luciferase fused to the C-
terminus of the fungal CBM did not show any significant
differences from cells expressing the native luciferase
Similarly, *in vitro* assays of the spectrum of bioluminescent
light emitted by a commercial source of purified recombinant
luciferase (Promega) were identical to the spectrum emitted by
the fusion protein.

The observed differences are therefore associated with the
concentrations of CoA. As the concentration of coenzyme A

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increases the spectral distribution alters and at the highest
concentrations of CoA the spectrum is dominated by wavelengths
in the region 590-630nm with a pronounced peak at 610nm. The
spectral shift is most marked for the double mutant where there
is a significant narrowing of bandwidth around a single peak of
wavelength 610nm (Figure 11).

Example 12
Production of synthetic Photinus pyralis luciferase mutated
such that it has 214C/354K/357F
A synthetic luc gene was designed and assembled from
oligonucleotide pairs using the synthesis strategy outlined
above. The gene sequence was engineered to create a luciferase
with the amino acids 214C, 354K and 357F.

Twenty-nine pairs of overlapping synthetic oligonucleotides
were synthesised by Sigma-Genosys Ltd, purified by PAGE and
ligated in three assemblies of approximately 550bp (IDRIS 1,2 &
3, Figure 13). Each assembly was then ligated separately into
the vector pBSK(+) and the resulting constructs were used to
transform E. coli XL1-Blue cells. Plasmid DNA was prepared from
clones containing the assembled inserts and sequenced to
confirm the fidelity of the ORFs. The presence of n-1
oligonucleotides (by-products of the oligosynthesis) in the
assemblies complicated the build process. DNA sequencing
identified a single correct assembly of IDRIS 2 and the PCR was
used to correct one assembly of IDRIS 3 which contained a
single base pair deletion at the 5' end of the construct.
Assembly of the complete ORF was achieved by ligating a mixture
of plasmids containing IDRIS 1 with IDRIS 2 and 3.

The ligated DNA was then used to transform E. coli XL1-Blue
cells and clones expressing active enzyme were selected using
an in vivo assay. Several clones were selected and sequenced to
confirm the presence and fidelity of the synthetic luc gene
having the sequence shown in Figure 14. The complete ORF was
called IDRIS (FA).

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The synthetic gene was assembled into the vector pBSK(+)
between the BamH I and Sal I sites in the polylinker. In this
position the gene is not in frame with the alpha peptide and is
a significant distance from the lac promoter. However, enough
5   luciferase is produced to enable preliminary characterisation
of the enzyme. Crude cell free extracts of *E. coli* XL1-Blue
cells expressing IDRIS (FA) were prepared, from overnight
cultures, using the Promega lysis method.

10   The thermostability of the enzyme in the extract was then
tested at 50°C over 20 minutes and compared with the
thermostable mutant E354I+D357Y. The new codon optimised triple
mutant was significantly more thermostable than the mutant
E354I+D357Y (Figure 15).

15

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Claims

1.   A recombinant protein having luciferase activity and at
least 60% similarity to a wild-type luciferase wherein in the
5   sequence of the enzyme, the amino acid residue corresponding to
residue 357 in *Photinus pyralis* luciferase is mutated as
compared to the corresponding wild-type luciferase, such that
the luciferase enzyme is able to emit light at a different
wavelength as compared to the corresponding wild-type
10   luciferase and/or has enhanced thermostability as compared to
the corresponding wild-type luciferase.

2.   A recombinant protein according to claim 1 wherein the
said wild-type luciferase sequence is of luciferase from
15   *Photinus pyralis, Luciola mingrelica, Luciola cruciata* or
Luciola lateralis, Hotaria paroula, Pyrophorus plagiophthalamus
Lampyris noctiluca, Pyrocoelia nayako or *Photinus*
pennsylvanica.

20   3.   A recombinant protein according to claim 2 wherein the
wild type luciferase sequence is the sequence of an enzyme
obtainable from *Photinus pyralis, Luciola mingrelica, Luciola*
cruciata or *Luciola lateralis* enzyme.

25   4.   A recombinant protein according to claim 1 or claim 2
wherein said wild-type luciferase sequence is of luciferase
from *Photinus pyralis, Luciola mingrelica, Luciola cruciata* or
Luciola lateralis, Hotaria paroula, Pyrophorus plagiophthalamus
Lampyris noctiluca or *Pyrocoelia nayako* and the amino acid
30   residue corresponding to residue 357 in *Photinus pyralis*
luciferase is other than aspartic acid or glutamic acid.

5.   A recombinant protein according to claim 1 or claim 2
wherein said wild-type luciferase sequence is of luciferase
35   from *Photinus pennsylvanica* and the amino acid residue
corresponding to residue 357 in *Photinus pyralis* luciferase is
other than valine.

6.   A recombinant protein according any one of the preceding
claims wherein the amino acid residue corresponding to residue
357 in *Photinus pyralis* luciferase is other than aspartic acid,
glutamic acid or valine.

5

7.   A recombinant protein according any one of the preceding
claims wherein the amino acid residue corresponding to residue
357 in *Photinus pyralis* luciferase is an uncharged polar amino
acid.

10

8.   A recombinant protein according to claim 7 wherein the
amino acid residue corresponding to residue 357 in *Photinus
pyralis* luciferase is tyrosine, phenylalanine or tryptophan.

15   9.   A recombinant protein according to claim 8 wherein the
amino acid residue corresponding to residue 357 in *Photinus
pyralis* luciferase is tyrosine.

10.   A protein according to any one of the preceding claims
20   wherein the protein has at least 80% similarity to luciferase
from *Photinus pyralis, Luciola mingrelica, Luciola cruciata* or
Luciola lateralis.

11.   A protein according to any one of the preceding claims
25   which has at least one of the following mutations as compared
to wild type luciferase:
(a) the amino acid residue corresponding to amino acid 354 of
the *Photinus pyralis* luciferase (356 in Luciola luciferase) is
mutated;
30   (b) the amino acid residue corresponding to position 215 in
Photinus pyralis luciferase or (217 in Luciola luciferase) is a
different hydrophobic amino acid;
(c) the amino acid residue corresponding to residue 214 in
Photinus pyralis luciferase or to residue 216 of *Luciola
35   mingrelica, Luciola cruciata* or *Luciola lateralis* luciferase;

(d) the amino acid residue corresponding to residue 232 in
Photinus pyralis luciferase or to residue 234 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis* luciferase;

(e) amino acid residue corresponding to residue 295 in *Photinus*
5   *pyralis* luciferase or to residue 297 of *Luciola mingrelica,*
Luciola cruciata or *Luciola lateralis* luciferase;

(f) amino acid residue corresponding to amino acid 14 of the
Photinus pyralis luciferase or to residue 16 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis;*

10   (g) amino acid residue corresponding to amino acid 35 of the
Photinus pyralis luciferase or to residue 37 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis;*

(h) amino acid residue corresponding to amino acid residue 105
of the *Photinus pyralis* luciferase or to residue 106 of *Luciola*
15   *mingrelica, Luciola cruciata* or *Luciola lateralis;*

(i) amino acid residue corresponding to amino acid residue 234
of the *Photinus pyralis* luciferase or to residue 236 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis;*

(j) amino acid residue corresponding to amino acid residue 420
20   of the *Photinus pyralis* luciferase or to residue 422 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis;*

(k) amino acid residue corresponding to amino acid residue 310
of the *Photinus pyralis* luciferase or to residue 312 of *Luciola*
mingrelica, Luciola cruciata or *Luciola lateralis;*

25   is different to the amino acid which appears in the
corresponding wild type sequence and wherein the luciferase
enzyme has increased thermostability as compared to an enzyme
having the amino acid of the corresponding wild-type luciferase
at this position.

30

12.   A protein according to any one of the preceding claims
wherein the amino acid residue corresponding to amino acid 354
of the *Photinus pyralis* luciferase (356 in Luciola luciferase)
is mutated.

35

13.   A protein according to claim 12 wherein the amino acid
residue corresponding to residue 214 in *Photinus pyralis*

luciferase or to residue 216 of *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis* luciferase is mutated to a different hydrophobic amino acid.

5   14. A nucleic acid which encodes a luciferase according to any one of the preceding claims.

15. A nucleic acid according to claim 14 which comprises a synthetic gene.

10

16. A nucleic acid according to claim 14 wherein the codon usage has been optimised for a particular expression host and/or unique restriction sites have been introduced.

15   17. A nucleic acid according to claim 14 or claim 15 which comprises nucleotides 9-1661 of SEQ ID NO 1, or a sequence which has at least 90% similarity thereto.

18. A vector comprising a nucleic acid according to any one of
20   claims 14 to 17.

19. A cell transformed with a vector according to claim 18.

20. A method of producing a protein according to any one of
25   claims 1 to 13, which method comprises culture of a cell according to claim 19.

21. The use of a protein according to any one of claims 1 to 13 in a bioluminescent assay.

30

22. A kit comprising a protein according to any one of claims 1 to 13.

23. A kit according to claim 22 which further comprises
35   luciferin.

24. An assay for determining the presence in a sample of CoA, which assay comprises adding to a sample suspected of

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containing CoA, luciferase as claimed in any one of claims 1 to
11 above together with other reagents which are required to
bring about a luciferase/luciferin reaction, measuring the
wavelenght of light emitted from the sample and relating this
5   to the presence or absence of CoA.

25.   An assay according to claim 24 for use in diagnosis of
diabetes.

Fig.1.

Fig.2(a).

Fig.2(b).

Fig.2(c).

Fig.2(d).

Fig.2(e).

Fig.2(f).

Fig.2(g).

Fig.2(h).

Fig.3.

Fig.4(a).

Fig.4(b).

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Fig.5.

Fig.6.

Fig.7a.

Fig.7b.

Fig.7c.

Fig.8(a).

Fig.8(b).

Fig.8(c).

Fig.9.

Fig.10.

Fig.11(a).

Fig.11(b).

Fig.12.

Fig.13.

IDRIS 1	BamH I	EcoR I	590bp	1-10
IDRIS 2	EcoR I	Kpn I	550bp	11-19
IDRIS 3	Kpn I	Sal I	555bp	20-29

Oligonucleotide pairs

Fig.14.(1/8)

```
                                                  BbeI
                                                  HaeII
                                                  HhaI|
                                                  NlaIV||
                                                  SfoI||
                                                  BsaHI|||
                                                  HinP1I|||
                                                  NarI|||
                                                  BanI||||
                                                  KasI||||
                                           HpaII ||||
                                           NciI  ||||
                                           ScrFI ||||
           DpnI                            BssKI | |||||
           NlaIV                           CviJI | |||||
BamHI |                                    HaeIII | |||||            MnlI
BstYI |             BbsI HgaI             Sau96I| | |||||             |
MboI  |   AlwI BsaHI |MboII                    || | |||||             |
      | |        |   | |    |                  || | |||||             |
      ggatccaaatggaagacgccaaaaacatcaagaaaggcccggcgccattctatcctctgg
   1  --------+---------+---------+---------+---------+---------+ 60
      cctaggtttaccttctgcggtttttgtagttctttccgggccgcggtaagataggagacc

  c       (I)(Q) M  E  D  A  K  N  I  K  K  G  P  A  P  F  Y  P  L  E -
                   ↑
                   BpmI
                   Cac8I
           FokI   |
           Cac8I| |
           MwoI|| |
           MspA1I||| |
           AciI |||| |                                          BstNI
BstF5I |   |||| |                                          ScrFI
NlaIV  | |||| |                                          BssKI |
BanI   | | |||| |                                         PspGI |
                                                          NlaIV| |
                                                    BstNI  || |
                                                    ScrFI  || |
                                                    BsaJI | ||| |
                                                    BssKI | ||| |
Mnl I  | | | ||||  |                               PspGI | ||| |
       | | | ||||  | HpyCH4V  CviJI    MaeIII      | || |
       aggatggcaccgctggcgagcaactgcataaggctatgaagcgttacgccctggttcctg
   61 --------+---------+---------+---------+---------+---------+ 120
       tcctaccgtggcgaccgctcgttgacgtattccgatacttcgaatgcgggaccaaggac

  c      D  G  T  A  G  E  Q  L  H  K  A  M  K  R  Y  A  L  V  P  G -
                                                      AciI
                                                      BstUI
                                                    RsaI |
                                                    TaiI |
```

Fig.14 (Cont).(2/8)

```
            SfaNI                                       Csp6I|  |
      MfeI    |                                          HphI|  |
    Tsp509I   |                                         BsaAI| |  |
      RsaI |  |                    MnlI                  BsiWI| |  |        BstBI
     Csp6I|  |        HpyCH4V     |   TaqI               MaeII| | |  |        TaqI
      | | |  |            |       |    |                    |||| |  |          |
      gtacaattgcttttacagatgcacatatcgaggtgaacatcacgtacgcggaatacttcg
121   ---------+---------+---------+---------+---------+---------+ 180
      catgttaacgaaaatgtctacgtgtatagctccacttgtagtgcatgcgccttatgaagc

 c      T   I   A   F   T   D   A   H   I   E   V   N   I   T   Y   A   E   Y   F   E -
```

```
                BstNI
                ScrFI
                BssKI |           AluI                   HphI
                PspGI |           CviJI             Hpy188I  |
                  | |             |                    |  |         Bst4CI
      aaatgtccgttcgcctggcagaagctatgaaacgctatggtctgaatacaaatcaccgta
181   ---------+---------+---------+---------+---------+---------+ 240
      tttacaggcaagcggaccgtcttcgatactttgcgataccagacttatgtttagtggcat

 c      M   S   V   R   L   A   E   A   M   K   R   Y   G   L   N   T   N   H   R   I -
```

```
                                                              HhaI
                                                             BstUI |
                                                             Cac8I |
                                                              HhaI |
                                                             HinP1I |
                                                              MwoI|  |
                        BtsI                                 BssHII|  |
             HpyCH4V    TspRI        EarI          HpaII     HinP1I|  |
             Hpy99I    |MboII  |    Tsp509I|       BsrFI|     BslI  |||  |
               |       | |     |        ||           ||         |   |||  |
      tcgtcgtatgcagtgaaaactctcttcaattctttatgccggtgctgggcgcgctttta
241   ---------+---------+---------+---------+---------+---------+ 300
      agcagcatacgtcacttttgagagaagttaagaaatacggccacgacccgcgcgaaaat

 c      V   V   C   S   E   N   S   L   Q   F   F   M   P   V   L   G   A   L   F   I -
```

```
                        Cac8I
                        NaeI
                      HpaII|
                      BsrFI| |
                       HhaI| |
                     NgoMIV| |
                      SgrAI| |
                     HinP1I ||                             TaiI        Bst4CI
             HpyCH4V       | |||          PsiI   MaeII      |     MseI    |
               |           | |||           |       |        |       |     |
      tcggtgttgcagttgcgccggcgaacgacatttataatgaacgtgaactgcttaacagta
301   ---------+---------+---------+---------+---------+---------+ 360
      agccacaacgtcaacgcggccgcttgctgtaaatattacttgcacttgacgaattgtcat

 c      G   V   A   V   A   P   A   N   D   I   Y   N   E   R   E   L   L   N   S   M -
```

```
                                                    ApoI
                                                  Tsp509I
```

Fig.14 (Cont).(3/8)

```
                                               HpyCH4V   |
                                               Cac8I   | |
                                               CviJI  | | |
              CviJI                            HaeIII | | | |
        Fnu4HI  |          BbvI              EcoO109I | | | |
        TseI|   |Bst4CI       |                Sau96I | | | |
          || |      |        |                       | | | |
          tgaacatttcgcagcctaccgtagtctttgtttccaaaaagggcctgcaaaaaattctca
    361 ---------+---------+---------+---------+---------+---------+ 420
          acttgtaaagcgtcggatggcatcagaaacaaaggtttttcccggacgtttttaagagt

  c      N  I  S  Q  P  T  V  V  F  V  S  K  K  G  L  Q  K  I  L  N -

        HpyCH4V                                        HinfI
          TaiI|                                          TfiI
    MaeII   ||                 Tsp509I       Tsp509I      NlaIII|
      |    |||                    |             |          ||
          acgtgcaaaaaaaactgccaattatccagaaaattattatcatggattctaaaacggatt
    421 ---------+---------+---------+---------+---------+---------+ 480
          tgcacgttttttttgacggttaataggtcttttaataatagtacctaagattttgcctaa

  c      V  Q  K  K  L  P  I  I  Q  K  I  I  I  M  D  S  K  T  D  Y -

                                   MaeIII
                                   Tsp45I
                                    TaiI |
                           MaeII    |  |
              CviJI        AflIII   |  | |
        BstNI   |            RsaI|  |  | |
        ScrFI   |           Csp6I|| |  | |             NciI
        BsaJI|  |           BsrGI||| |  | |            ScrFI
        BssKI|| |            TatI||| |  | |            HpaII|      MnlI
        PspGI|| |     TaqI       ||| |  | |            BssKI||     MseI
          |||  |       |     ||||  | || |              |||        |
          accagggctttcagtcgatgtacacgttcgtcacatctcatctgcctccgggttttaatg
    481 ---------+---------+---------+---------+---------+---------+ 540
          tggtcccgaaagtcagctacatgtgcaagcagtgtagagtagacggaggcccaaaattac

  c      Q  G  F  Q  S  M  Y  T  F  V  T  S  H  L  P  P  G  F  N  E -

                                                            ApoI
                                                            EcoRI
                                                            NlaIII
                                       MaeIII               Tsp509I
                                       Tsp45I                BspHI    |
                                       DpnI  |               TspRI    |
                                       MlyI| |       HpyCH4V  DpnI|    |
                    RsaI               MboI|| |        MfeI  |BclI ||  |
                    Csp6I|HinfI        PleI|| |      Tsp509I |MboI ||  |
                      ||   |           |||  |          |     |  | ||  |
          aatacgattttgtaccagagtcctttgatcgtgacaaaacaattgcactgatcatgaatt
    541 ---------+---------+---------+---------+---------+---------+ 600
          ttatgctaaaacatggtctcaggaaactagcactgttttgttaacgtgactagtacttaa

  c      Y  D  F  V  P  E  S  F  D  R  D  K  T  I  A  L  I  M  N  S -

                                                            Cac8I
```

Fig.14 (Cont).(4/8)

```
                                              CviJI            SfaNI   |
           MnlI        Bsu36I         HaeIII        MslI    |  |
  CviJI      |    BsrI DdeI      Sau96I|   AciI HgaI   |    |  |
    |        |     |    |          ||      |     |     |    |  |
  cctctggctctactggtctgcctaagggtgtggcccttccgcatcgttgtgcctgcgtcc
601 ---------+---------+---------+---------+---------+---------+ 660
  ggagaccgagatgaccagacggattcccacaccgggaaggcgtagcaacacggacgcagg

c     S   G   S   T   G   L   P   K   G   V   A   L   P   H   R   C   A   C   V   R  -
```

```
                      FauI
               DpnI  |
               MboI  |  |
             BstUI|  |  |
             AciI ||  |  |
             Cac8I||  |  |
             AlwI |  || |  |
             NlaIII|  || |  |                      HpaII
             NspI |  || |  |      BstEII            BsaWI|              DdeI
             SphI |  || |  |      MaeIII            BspEI|     HinfI     |
             Cac8I|  || |  |      BslI  |     BciVI  ||BseMII Tfil   |
              |   |  ||| | |       |    |       |    ||  |     |    |   |
  gtttctcgcatgccgcgatcctatttttggtaaccaaatcattccggatactgcgattc
661 ---------+---------+---------+---------+---------+---------+ 720
  caaagagcgtacgggcgctaggataaaaaccattggtttagtaaggcctatgacgctaag

c     F   S   H   A   R   D   P   I   F   G   N   Q   I   I   P   D   T   A   I   L  -
```

```
                          BstXI
                   NlaIII  |
                   XcmI    |
               BsaJI |     |
               BtgI  |     |
               NcoI  |     |      NlaIII                   DpnI
  Hpy188I      StyI  |     |      NspI      CviJI   Hpy188I Mbol |
    |            |   |     |       |          |       |      |  | |
  tgagtgttgttccattccaccatggttttggcatgtttactacactcggctatctgatct
721 ---------+---------+---------+---------+---------+---------+ 780
  actcacaacaaggtaaggtggtaccaaaaccgtacaaatgatgtgagccgatagactaga

c     S   V   V   P   F   H   H   G   F   G   M   F   T   T   L   G   Y   L   I   C  -
```

```
                                                      HhaI
                                              HinP1I  |
                                              MboII   |  |
                                              MwoI|   |  |
                              EarI            MboII ||  |  |HpyCH4V
                      MnlI    |       AluI    |  ||  |  |  SfcI  |
  CviJI               NlaIII  |SapI   CviJI   |  ||  |  |  |MwoI | |
    |                   |     | |  |     |    |  || |   | |  || | |
  gtggctttcgtgtcgtcctcatgtatcgctttgaagaagagctgtttctgcgctccctgc
781 ---------+---------+---------+---------+---------+---------+ 840
  caccgaaagcacagcaggagtacatagcgaaacttcttctcgacaaagacgcgagggacg

c     G   F   R   V   V   L   M   Y   R   F   E   E   E   L   F   L   R   S   L   Q  -
                                 NlaIV
```

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Fig.14 (Cont).(5/8)

```
   PstI        ApoI              HhaI  BanI  |
   SbfI     Tsp509I            HinP1I  |     |      MboII
    |          |                 |  |  |     |       |
          aggattacaaaattcaaagtgcgcttctggtgccaaccctgttttcattcttcgccaaaa
   841    ---------+---------+---------+---------+---------+---------+  900
          tcctaatgttttaagtttcacgcgaagaccacggttgggacaaaagtaagaagcggtttt

   c      D   Y   K   I   Q   S   A   L   L   V   P   T   L   F   S   F   F   A   K   S  -
```

```
                                                                           BseSI
                                                                          BsiHKAI
                                                                         Bsp1286I
                                                                         HpyCH4V  |
                                                                          ApaLI |  |
                                                                          AciI |  |  |
                                                              Fnu4HI |  |  |
                                                               AciI|  |  |
                                                              Cac8I ||  |  |
                                       MboII                   BcgI|  |  |  |
                                       DpnI  |                 BfaI|  |  |  |
                   Hpy188I             MboI  |  |        Tsp509I  NheI||  ||  |  |  |
                      |                 |  |  |              |       |||  ||  |  |  |
          gcactctgattgacaaatacgatctgtctaatcttcacgaaattgctagcggcggtgcac
   901    ---------+---------+---------+---------+---------+---------+  960
          cgtgagactaactgtttatgctagacagattagaagtgctttaacgatcgccgccacgtg

   c      T   L   I   D   K   Y   D   L   S   N   L   H   E   I   A   S   G   G   A   P  -
                                                                              '315
```

```
                                                          BstF5I
                                                    BstNI  |
               MnlI                                 ScrFI  |
          BstBI  |           BcgI    HpyCH4V    FokI   BssKI  |  |
          TaqI   |  Hpy188I  |AciI     |   MboII  |   PspGI |  |
           |     |     |    |  |  |    |     |    |     |  |  |  |
          ctctttcgaaagaagtcggagaagcggttgcaaaacgcttccatcttccaggcatccgtc
   961    ---------+---------+---------+---------+---------+---------+  1020
          gagaaagctttcttcagcctcttcgccaacgttttgcgaaggtagaaggtccgtaggcag

   c      L   S   K   E   V   G   E   A   V   A   K   R   F   H   L   P   G  [I  R]  Q  -
```

```
                       TspRI
                DdeI     |
          BseMII   BsaI|     |
          CviJI  |   BsmAI|     |  BfaI
          SfaNI  |  |BsmAI  ||   |SpeI|        Hpy188I
           |     |  |  |    |  || |  ||            |
          aaggctatggtctcactgagactactagtgctattctgattacaccgaagggcgatttca
   1021   ---------+---------+---------+---------+---------+---------+  1080
          ttccgataccagagtgactctgatgatcacgataagactaatgtggcttcccgctaaagt

   c      G   Y   G   L   T   E   T   T   S   A   I   L   I   T   P   K   G   D   F   K  -
                             |
                BsiEI        345
             AciI   |
             BstUI  |
             HhaI   |             KpnI
          HinP1I |  |          NlaIV |
```

Fig.14 (Cont).(6/8)

```
         NciI    | |     |      RsaI  |                    DpnI
         ScrFI   | |     |      Csp6I |  |               BciVI  |
         HpaII|  | |     |      Acc65I|  |               BstYI  |     BsrFI
         BssKI|| | |     |      BanI  |  |               MboI   |     AlwI  |
            ||| | |     |            |||  |                  | |        | |
         aaccgggcgcggtcggtaaagtggtaccattttttgaagcgaaggttgtggatctggata
1081     ---------+---------+---------+---------+---------+---------+ 1140
         ttggcccgcgccagccatttcaccatggtaaaaaacttcgcttccaacacctagacctat

c         P  G  A  V  G  K  V  V  P  F  F  E  A  K  V  V  D  L  D  T -
```

```
                                                         AvaII
                                                         Sau96I
                                                         SacII|
                                                         AciI||
                                                         BstUI||
                                                         MspA1I||
                                                         AciI |||
                                                         BsaJI|||
         HpaII              MseI           Bst4CI    BtgI |||
           |                 |               |        | |||
         ccggcaaaacgctgggcgttaatcagcgtggcgaactgtgtgtccgcggtcctatgatta
1141     ---------+---------+---------+---------+---------+---------+ 1200
         ggccgttttgcgacccgcaattagtcgcaccgcttgacacacaggcgccaggatactaat

c         G  K  T  L  G  V  N  Q  R  G  E  L  C  V  R  G  P  M  I  M -
```

```
                                                         Fnu4HI
                                                         CviJI|
                                                         TseI|
                            HpaII                        Cac8I ||
         HpaII              BsaWI|                       BstF5I| ||
         BsaWI|             BspEI|          MwoI    BbvI CviJI|| ||
            ||                ||             |       |      ||| |||
         tgtccggttatgtaaacaatccggaagcgaccaacgcccttattgacaaggatggctggc
1201     ---------+---------+---------+---------+---------+---------+ 1260
         acaggccaatacatttgttaggccttcgctggttgcgggaataactgttcctaccgaccg

c         S  G  Y  V  N  N  P  E  A  T  N  A  L  I  D  K  D  G  W  L -
```

```
                                              MboII
                                       BsmFI   |
                                       BbsI  | |
         FokI                          MboII | | |          AciI
         BsmI|                   BsrI Bmr I | | | |         HincII   |
         HpyCH4V|                 |    |    | | | |          |       | |
            ||                    |    | | | | | |          |       | |
         tgcattctggcgacatcgcttactgggacgaagacgaacacttcttcatcgttgaccgcc
1261     ---------+---------+---------+---------+---------+---------+ 1320
         acgtaagaccgctgtagcgaatgaccctgcttctgcttgtgaagaagtagcaactggcgg

c         H  S  G  D  I  A  Y  W  D  E  D  E  H  F  F  I  V  D  R  L -
```

```
                            BcgI              DpnI
                       AluI  |           MboI  |
                       CviJI |           ClaI| |
                       MspA1I|           TaqI| |
                 NlaIV      | |          BsrI || |
```

Fig.14 (Cont).(7/8)

```
                                          CviJI|      |  |  HinfI|  ||| |
               MseI          CviJI        HaeIII|     |  |   TfiI|  ||| |
         BsmAI   |    Eco57I    |          Sau96I|| |PvuII |  AlwI||  ||| |
           |     |      |       |              ||| |    |  |     ||| || |
         tgaagtctctcattaaatacaaaggctatcaggtggccccagctgaactggaatcgatcc
   1321  ---------+---------+---------+---------+---------+---------+ 1380
         acttcagagagtaatttatgtttccgatagtccaccggggtcgacttgaccttagctagg

c       K   S   L   I   K   Y   K   G   Y   Q   V   A   P   A   E   L   E   S   I   L -
```

```
                                      BbsI
                                      HgaI
                                      MboII
                              Cac8I     |
                              AciI |    |
                              BspMI|    |
                              BstUI|    |
                              Hpy99I|   |                          HpaII
               MboII        TaqI  | |   |              HpaII       BsrFI|
               MnlI  |      FauI  | |   |              BsaWI|      SgrAI|
         HpyCH4V  | |      BcgI | | |   | |MwoI        BspEI|      BsaHI ||
           |      | |       |   | | |   | |  |             ||          | ||
         tcctgcaacacccaaacatcttcgacgcgggcgtggcaggtcttccggacgatgacgccg
   1381  ---------+---------+---------+---------+---------+---------+ 1440
         aggacgttgtgggtttgtagaagctgcgcccgcaccgtccagaaggcctgctactgcggc

c       L   Q   H   P   N   I   F   D   A   G   V   A   G   L   P   D   D   D   A   G -
```

```
               AciI
               BsiEI
               Fnu4HI
               CviJI|                    Bst4CI
               HaeIII|           BsiHKAI   |
               HphI|             Bsp1286I  |
               EaeI ||            TaqI  |  |
               EagI ||            AvaI| |  |
         HgaI HpaII| ||           SmlI| |  |              DpnI
           |    || ||             XhoI| |  |              MboI  |
           |    || ||               || |  |                 |  |
         gtgaacttccggccgccgttgttgttctcgagcacggtaagacgatgacggaaaaagaga
   1441  ---------+---------+---------+---------+---------+---------+ 1500
         cacttgaaggccggcggcaacaacaagagctcgtgccattctgctactgccttttttctct

c       E   L   P   A   A   V   V   V   L   E   H   G   K   T   M   T   E   K   E   I -
```

```
                                               AciI
                                               BstUI
                                               HhaI
                                   BsrI         HinP1I |
                          Hpy99I|              Fnu4HI | |
                          TaiI ||       BstUI  AluI|  | |
                  MaeII   |  ||    BcgI  AciI | CviJI| | |
                   |      | |||  MaeIII Bbvl | |  TseI| | |          BcgI
                   |      | |||    |    | | | | |   || | |            |
         tcgtggattacgtcgccagtcaagtaacaaccgcgaaaaagctgcgcggtggcgttgtgt
   1501  ---------+---------+---------+---------+---------+---------+ 1560
         agcacctaatgcagcggtcagttcattgttggcgcttttttcgacgcgccaccgcaacaca
```

Fig.14 (Cont).(8/8)

```
c       V  D  Y  V  A  S  Q  V  T  T  A  K  K  L  R  G  G  V  V  F -

                                                      HgaI          DpnI
                                                   TaiI|        BstYI  |
                                             BsaAI | |         MboI   |
                                             MaeII|  | |     BstUI   |  |
            RsaI              HpaII         Hpy99I || ||   AciI  |  |  |
            Csp6I|            BsrFI|     TaqI   | || ||   AlwI |  |  |  |
               ||                ||          |  | || ||       |  |  |  |
            ttgtggacgaagtaccgaaaggtcttaccggcaaactcgacgcacgtaaaatccgcgaga
      1561  ---------+---------+---------+---------+---------+---------+ 1620
            aacacctgcttcatggctttccagaatggccgtttgagctgcgtgcattttaggcgctct

c       V  D  E  V  P  K  G  L  T  G  K  L  D  A  R  K  I  R  E  I -

                 CviJI
                 HaeIII                      MseI
              BslI   |                     AluI  |
              MseI|  |                     CviJI |
              EcoNI| |MnlI      AciI    HindIII |  |
                 ||| |   |        |         |   |  |
            tcctcattaaggccaagaagggcggtaagtccaagctttaa
      1621  ---------+---------+---------+---------+- 1661
            aggagtaattccggttcttcccgccattcaggttcgaaatt

c       L  I  K  A  K  K  G  G  K  S  K  L  * -
```

Fig.15.

Inactivation of mutants E354I+D357Y and IDRIS
(FA) at 50C

♦ IY

■ IDRIS (FA)

SEQUENCE LISTING

<110> The Secretary of State for Defence
      White, Peter J
      Willey, Tara L
      Price, Rachel L
      Murphy, Melanie J
      Squirrell, David

<120> Novel Enzyme

<130> DERA/IPD/P1247/WOD

<140>
<141>

<150> GB 9925161.3
<151> 1999-10-26

<150> GB 0016744.5
<151> 2000-07-10

<160> 13

<170> PatentIn Ver. 2.1

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<211> 1661
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (3)..(1661)

<220>
<223> Description of Artificial Sequence: cDNA sequence
      of the synthetic luciferase gene

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gg atc caa atg gaa gac gcc aaa aac atc aag aaa ggc ccg gcg cca   47
   Ile Gln Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro
       -1   1   5   10

ttc tat cct ctg gag gat ggc acc gct ggc gag caa ctg cat aag gct   95
Phe Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala
    15   20   25

atg aag cgt tac gcc ctg gtt cct ggt aca att gct ttt aca gat gca   143
Met Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala
30   35   40   45

cat atc gag gtg aac atc acg tac gcg gaa tac ttc gaa atg tcc gtt   191
His Ile Glu Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val
                    50   55   60

cgc ctg gca gaa gct atg aaa cgc tat ggt ctg aat aca aat cac cgt   239
Arg Leu Ala Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg
                65   70   75

```
atc gtc gta tgc agt gaa aac tct ctt caa ttc ttt atg ccg gtg ctg    287
Ile Val Val Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu
            80              85              90

ggc gcg ctt ttt atc ggt gtt gca gtt gcg ccg gcg aac gac att tat    335
Gly Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr
    95              100             105

aat gaa cgt gaa ctg ctt aac agt atg aac att tcg cag cct acc gta    383
Asn Glu Arg Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val
110             115             120             125

gtc ttt gtt tcc aaa aag ggc ctg caa aaa att ctc aac gtg caa aaa    431
Val Phe Val Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys
            130             135             140

aaa ctg cca att atc cag aaa att att atc atg gat tct aaa acg gat    479
Lys Leu Pro Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp
            145             150             155

tac cag ggc ttt cag tcg atg tac acg ttc gtc aca tct cat ctg cct    527
Tyr Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro
            160             165             170

ccg ggt ttt aat gaa tac gat ttt gta cca gag tcc ttt gat cgt gac    575
Pro Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp
    175             180             185

aaa aca att gca ctg atc atg aat tcc tct ggc tct act ggt ctg cct    623
Lys Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro
190             195             200             205

aag ggt gtg gcc ctt ccg cat cgt tgt gcc tgc gtc cgt ttc tcg cat    671
Lys Gly Val Ala Leu Pro His Arg Cys Ala Cys Val Arg Phe Ser His
            210             215             220

gcc cgc gat cct att ttt ggt aac caa atc att ccg gat act gcg att    719
Ala Arg Asp Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile
            225             230             235

ctg agt gtt gtt cca ttc cac cat ggt ttt ggc atg ttt act aca ctc    767
Leu Ser Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu
            240             245             250

ggc tat ctg atc tgt ggc ttt cgt gtc gtc ctc atg tat cgc ttt gaa    815
Gly Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu
    255             260             265

gaa gag ctg ttt ctg cgc tcc ctg cag gat tac aaa att caa agt gcg    863
Glu Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala
270             275             280             285

ctt ctg gtg cca acc ctg ttt tca ttc ttc gcc aaa agc act ctg att    911
Leu Leu Val Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile
            290             295             300

gac aaa tac gat ctg tct aat ctt cac gaa att gct agc ggc ggt gca    959
Asp Lys Tyr Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala
            305             310             315

cct ctt tcg aaa gaa gtc gga gaa gcg gtt gca aaa cgc ttc cat ctt    1007
```

```
        Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu
                320                 325                 330

cca ggc atc cgt caa ggc tat ggt ctc act gag act act agt gct att      1055
Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile
        335                 340                 345

ctg att aca ccg aag ggc gat ttc aaa ccg ggc gcg gtc ggt aaa gtg      1103
Leu Ile Thr Pro Lys Gly Asp Phe Lys Pro Gly Ala Val Gly Lys Val
350                 355                 360                 365

gta cca ttt ttt gaa gcg aag gtt gtg gat ctg gat acc ggc aaa acg      1151
Val Pro Phe Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr
                370                 375                 380

ctg ggc gtt aat cag cgt ggc gaa ctg tgt gtc cgc ggt cct atg att      1199
Leu Gly Val Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile
                385                 390                 395

atg tcc ggt tat gta aac aat ccg gaa gcg acc aac gcc ctt att gac      1247
Met Ser Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp
        400                 405                 410

aag gat ggc tgg ctg cat tct ggc gac atc gct tac tgg gac gaa gac      1295
Lys Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp
        415                 420                 425

gaa cac ttc ttc atc gtt gac cgc ctg aag tct ctc att aaa tac aaa      1343
Glu His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys
430                 435                 440                 445

ggc tat cag gtg gcc cca gct gaa ctg gaa tcg atc ctc ctg caa cac      1391
Gly Tyr Gln Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His
                450                 455                 460

cca aac atc ttc gac gcg ggc gtg gca ggt ctt ccg gac gat gac gcc      1439
Pro Asn Ile Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala
                465                 470                 475

ggt gaa ctt ccg gcc gcc gtt gtt gtt ctc gag cac ggt aag acg atg      1487
Gly Glu Leu Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met
        480                 485                 490

acg gaa aaa gag atc gtg gat tac gtc gcc agt caa gta aca acc gcg      1535
Thr Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala
        495                 500                 505

aaa aag ctg cgc ggt ggc gtt gtg ttt gtg gac gaa gta ccg aaa ggt      1583
Lys Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly
510                 515                 520                 525

ctt acc ggc aaa ctc gac gca cgt aaa atc cgc gag atc ctc att aag      1631
Leu Thr Gly Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys
                530                 535                 540

gcc aag aag ggc ggt aag tcc aag ctt taa                              1661
Ala Lys Lys Gly Gly Lys Ser Lys Leu
                545                 550
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<210> 2
<211> 552
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Encoded amino acid
      sequence of the cDNA sequence of synthetic
      luciferase gene

<400> 2
Ile Gln Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe
    -1   1             5                  10
Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met
15               20                  25                      30
Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His
            35                  40                  45
Ile Glu Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg
            50                  55                  60
Leu Ala Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile
        65                  70                  75
Val Val Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly
    80                  85                  90
Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn
95                  100                 105                 110
Glu Arg Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val
            115                 120                 125
Phe Val Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys
            130                 135                 140
Leu Pro Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr
            145                 150                 155
Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro
    160                 165                 170
Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys
175                 180                 185                 190
Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys
            195                 200                 205
Gly Val Ala Leu Pro His Arg Cys Ala Cys Val Arg Phe Ser His Ala
            210                 215                 220
Arg Asp Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu
        225                 230                 235
Ser Val Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly
    240                 245                 250
Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu
255                 260                 265                 270
Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu
            275                 280                 285
Leu Val Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp
            290                 295                 300
Lys Tyr Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro
        305                 310                 315
Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro
    320                 325                 330
Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu
335                 340                 345                 350
Ile Thr Pro Lys Gly Asp Phe Lys Pro Gly Ala Val Gly Lys Val Val
            355                 360                 365
Pro Phe Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu
            370                 375                 380
Gly Val Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met
        385                 390                 395
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Ser Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys
    400             405             410
Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu
415             420             425                     430
His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly
            435             440             445
Tyr Gln Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro
        450             455             460
Asn Ile Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly
        465             470             475
Glu Leu Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr
    480             485             490
Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys
495             500             505                     510
Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu
            515             520             525
Thr Gly Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala
        530             535             540
Lys Lys Gly Gly Lys Ser Lys Leu
        545             550
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<210> 3
<211> 1661
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: cDNA sequence
      of synthetic luciferase gene

<400> 3
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ttaaagcttg gacttaccgc ccttcttggc cttaatgagg atctcgcgga ttttacgtgc 60
gtcgagtttg ccggtaagac ctttcggtac ttcgtccaca aacacaacgc caccgcgcag 120
cttttttcgcg gttgttactt gactggcgac gtaatccacg atctcttttt ccgtcatcgt 180
cttaccgtgc tcgagaacaa caacggcggc cggaagttca ccggcgtcat cgtccggaag 240
acctgccacg cccgcgtcga agatgtttgg gtgttgcagg aggatcgatt ccagttcagc 300
tggggccacc tgatagcctt tgtatttaat gagagacttc aggcggtcaa cgatgaagaa 360
gtgttcgtct tcgtcccagt aagcgatgtc gccagaatgc agccagccat ccttgtcaat 420
aagggcgttg gtcgcttccg gattgtttac ataaccggac ataatcatag gaccgcggac 480
acacagttcg ccacgctgat taacgcccag cgttttgccg gtatccagat ccacaacctt 540
cgcttcaaaa aatgttaccga ctttaccgac cgcgcccggt ttgaaatcgc ccttcggtgt 600
aatcagaata gcactagtag tctcagtgag accatagcct tgacggatgc ctggaagatg 660
gaagcgtttt gcaaccgctt ctccgacttc tttcgaaaga ggtgcaccgc cgctagcaat 720
ttcgtgaaga ttagacagat cgtatttgtc aatcagagtc ctttttgcga agaatgaaaa 780
cagggttggc accagaagcg cactttgaat tttgtaatcc tgcagggagc gcagaaacag 840
ctcttcttca aagcgataca tgaggacgac acgaaagcca cagatcagat agccgagtgt 900
agtaaacatg ccaaaaccat ggtggaatgg aacaacactc agaatcgcag tatccggaat 960
gatttggtta ccaaaaatag gatcgcgggc atgcgagaaa cggacgcagg cacaacgatg 1020
cggaagggcc acacccttag gcagaccagt agagccagag gaattcatga tcagtgcaat 1080
tgttttgtca cgatcaaagg actctggtac aaaatcgtat tcattaaaac ccggaggcag 1140
atgagatgtg acgaacgtgt acatcgactg aaagccctgg taatccgttt tagaatccat 1200
gataataatt ttctggataa ttggcagttt ttttttgcacg ttgagaattt tttgcaggcc 1260
cttttttggaa acaaagacta cggtaggctg cgaaatgttc atactgttaa gcagttcacg 1320
ttcattataa atgtcgttcg ccggcgcaac tgcaacaccg ataaaaagcg cgcccagcac 1380
cggcataaag aattgaagag agttttcact gcatacgacg atacggtgat ttgtattcag 1440
accatagcgt ttcatagctt ctgccaggcg aacggacatt tcgaagtatt ccgcgtacgt 1500
gatgttcacc tcgatatgtg catctgtaaa agcaattgta ccaggaacca gggcgtaacg 1560
cttcatagcc ttatgcagtt gctcgccagc ggtgccatcc tccagaggat agaatggcgc 1620
cgggcctttc ttgatgtttt tggcgtcttc catttggatc c                     1661
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<210> 4
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 4
cacccgaggg ggattataaa ccgggcgcgg                          30


<210> 5
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 5
cacccgaggg ggatvycaaa ccgggcgcgg tcgg                     34


<210> 6
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 6
cacccgaggg ggattdsaaa ccgggcgcgg tcgg                     34


<210> 7
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 7
cacccgaggg ggatmrsaaa ccgggcgcgg tcgg                     34


<210> 8
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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Oligonucleotide

<400> 8
gggctcactg agactactag tgctattatg attacacccg   40

<210> 9
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 9
cgggtgtaat cagaatagca ctagtagtct cagtgagccc   40

<210> 10
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 10
ggcgcggtcg gtaaagtggt accatttttt gaagcg   36

<210> 11
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide

<400> 11
cgcttcaaaa aatggtacca ctttaccgac cgcgcc   36

<210> 12
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (24)..(25)
<223> n=a or g or c or t

<220>
<221> misc_feature
<222> (33)..(34)
<223> n=a or g or c or t

<220>

<223> Description of Artificial Sequence:
     Oligonucleotide

<400> 12
ctagtgctat tctgattaca cccnnsgggg atnnsaaacc gggcgcggtc ggtaaagtgg 60
ta   62

<210> 13
<211> 55
<212> DNA
<213> Artificial Sequence

<220>
<221> misc_feature
<222> (26)..(27)
<223> n=a or g or c or t

<220>
<221> misc_feature
<222> (35)..(36)
<223> n=a or g or c or t

<220>
<223> Description of Artificial Sequence:
     Oligonucleotide

<400> 13
cactttaccg accgcgcccg gtttsnnatc cccsnngggt gtaatcagaa tagca   55

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